

Are world summits really necessary?

The widely forecast disappointment at last week's meeting of the G7 countries in Halifax, Nova Scotia, is simply explained: governments choose to talk about the wrong things, and they are the wrong governments anyway.

SHOULD the rich countries of the world continue meeting once a year, as they did at Halifax in Nova Scotia last week, when the outcomes of their deliberations appear perennially inconclusive? This is an old problem. The rich countries (called the 'G7' countries, although there are really eight of them, with President Boris Yeltsin of Russia now a regular participant) usually have nothing substantial to say after their annual meetings. Or if they manage to find common ground, they may commit themselves to some common action and, afterwards, discover that they do not have the stomach to carry it through. This year's meeting seems to have been a particular disappointment: the decision that the International Monetary Fund (IMF) should have resources to avoid a repetition of last year's financial crisis in Mexico will be useful (if members of that organization stump up the extra funds), but that could have been agreed at the IMF's own meeting in Washington just a few weeks ago. Otherwise, nothing much appears to have been decided. No wonder many of the participants are grumbling (not for the first time) that their journeys have been unnecessary.

Is that really so? Last week's proceedings show all too clearly that the people who assemble talk about all the wrong things. In advance of Halifax, for example, the United States and Japan had let it be known that they would not spoil the meeting by arguing out their dispute about the bilateral trade in motor-cars; in the event, they appear to have talked about it, but mostly to (or at) each other. Yet this bilateral dispute is potentially a means by which the international trading system, and the new World Trade Organization (the successor to the General Agreement on Tariffs and Trade, or GATT), could be permanently undermined, to the general discomfort of the whole world. These are precisely the circumstances, those of a strictly bilateral dispute with serious international repercussions, that should have been firmly on the agenda at Halifax. By what right did the two participants seek to relegate it to the corridors?

By contrast, Bosnia, by all accounts promoted to the Halifax agenda at the urgent insistence of President Jacques Chirac of France, would have been more aptly left aside. The world community, and the G7 subset thereof, has been wringing its hands for four years at the spectacle of an apparently unstoppable conflict in a region that should by now be part of a peaceful Europe. Some useful work has been done by the United Nations forces stationed in the area; the contingency arrangements put in place to deal with a more serious escalation of the conflict may also

yet prove invaluable. But everybody knows there is no chance that the US president will be allowed by a Congress dominated by his political opponents to commit ground forces to peacekeeping in Bosnia while there is no clear strategy for bringing the conflict to an end. Raising the issue at Halifax will not have changed that state of affairs, but may unhelpfully have served to make President Bill Clinton seem less powerful than he is. How does that serve G7's interest?

The other glaring defect of the G7 procedure is its invitation list. Russia originally gatecrashed the club by arguing that its need of financial assistance compelled its presence, and seems to have stayed on the list. But there are conspicuous omissions, of which China is the most conspicuous. At the outset 22 years ago, the rationale for G7 was that its members collectively held the balance of economic power in the world, so that their agreed decisions on matters in and even outside the strictly economic field would probably carry weight with those not invited to the meetings. But China is already a substantial economic power, and is likely quickly to become even stronger. Moreover, China seems to take delight in declining to heed decisions in whose making it has played no part — witness the endless arguments over the details of the transition of Hong Kong from British colony to Chinese province. The present members of G7 would no doubt find a Chinese presence at their meetings uncomfortable, but it is difficult to see how those meetings can be made more effective if China is not eventually asked along. □

Proliferation and muddle

One of the most imaginative of overseas initiatives by the United States is needlessly in trouble.

WHAT is to happen to all the enriched uranium that used to be locked up in nuclear warheads built by the former Soviet Union, and due to be dismantled under international agreements? The obvious danger is that it will find its way into the hands of governments wishing to make nuclear weapons. That danger was clearly apparent four years ago. To its credit, the Bush administration in the United States then hit on a scheme for neutralizing a potentially hair-raising threat: the United States would buy the ex-Soviet uranium, dilute it with non-fissile isotopes and use it as



commercial reactor fuel. A total of \$500 million was set aside to help with the dismantling of Russian warheads, and a commercial price was fixed for the enriched uranium. Calamity appeared to have been averted.

But now this imaginative deal may be falling apart. A first shipment of uranium is due soon, but reluctantly; the Russian government is apparently having second thoughts about the price at which its enriched uranium will be sold. Part of the explanation is that the customer in the United States is not the US government, but a corporation created (and still owned) by the government with the solid-sounding name of the US Enrichment Corporation. The plan is that the company will manage not only the conversion of the Russian weapons-grade uranium into commercial fuel, but will also take over from the US Department of Energy and operate the enrichment facilities in the United States that, for half a century, have produced enriched uranium for US bombs. Eventually, the enrichment corporation will be sold into the private sector.

The US government seems to have been surprised by one consequence of this state of affairs: the enrichment company, being a commercial entity, is naturally subject to US laws on commercial trade. In particular, domestic producers of uranium are free to seek redress from the US Department of Commerce if they believe uranium is being dumped on the US market at too low a price. This is precisely what the US uranium-mining industry did in 1992, since when the Russian deal has been caught up in the bureaucracy applicable to classical trade disputes. It seems not to matter much to anybody that uranium mining in the United States is no longer a booming activity, but an industry employing just a few hundred people. A further difficulty is that the cost to the enrichment company of enriching uranium at domestic plants (which have been transferred to it at negligible cost) is less than it will have to pay for the Russian material. The result is that where once, just four years ago, there was a willing buyer and a willing seller, now both parties to the deal on Russian enriched fuel appear to have become reluctant participants.

For the past two decades, the United States has been stalwart in seeking to prevent the spread of nuclear weapons. It may have been over-zealous in seeking to prevent the extraction of plutonium from spent reactor fuel, but generally its influence has been enlightened and powerful. Who can guess where along the road to nuclear power Iraq and North Korea would now have been had it not been for US intervention? The United States has also faced down military opinions that weapons-testing should continue for the sake of winning agreement to a comprehensive ban on nuclear tests some time next year. (If only France and China would follow suit!) Yet the deal on ex-Soviet bomb-making material is potentially a more tangible contribution to the anti-proliferation cause than any other, and is in difficulties for largely administrative reasons. The US administration has other things on its mind, to be sure, but the opportunity to buy up the world's largest stock of enriched uranium deserves more attention than it has been given. And quickly. □

How to police fraud

Only academic institutions can effectively impose sanctions on those found guilty of scientific misconduct.

REACTION in Britain to the serious case of scientific misconduct at St George's Hospital is in danger of getting out of hand (see *Nature* 375, 522 & 529; 1995). Not that the facts are in dispute. A published report of the transplantation of an ectopic pregnancy into the uterus where it properly belonged was found untrue, and the surgeon primarily responsible for the publication was reported to the professional licensing authority (the General Medical Council, or GMC) and has now lost his licence to practice (but is appealing against the decision). On the face of things, St George's Hospital acted promptly and properly in dealing with the trouble, although it would have been better advised to make public the names of those appointed to carry out its internal investigation. But now the Royal College of Physicians in London plans a consultation with similar organizations on whether there should be a central body to coordinate investigations into allegations of scientific fraud.

Nothing in what follows should be construed as a suggestion that fraud in science is anything but an abomination, a means by which the mutual trust of colleagues is corroded and the literature corrupted. Nor is it in question that sanctions of some kind should be applied to those found guilty of misconduct. The practical difficulty is that only people's employers are in a position to impose sanctions on transgressors. So much should by now be clear from experience in the United States. The elaborate procedures by which the National Institutes of Health (NIH) and their sponsoring department look into allegations of misconduct yield, when successful, a mouse of a sanction. Those found guilty may be barred from applying for research grants for a certain period; they cannot be dismissed from their jobs, because they are not employed by the NIH.

In Britain, a "central body" would be a toothless animal unless it were established by statute. But what would the legal draughtsmen make of the concepts of "scientific misconduct" and of "scientist"? And how could the research community live comfortably with the results of any definitions, which would certainly be over-sharp and probably an infringement of freedom in research as well?

That is why the best that the physicians can hope for is a central body that does not have investigatory powers and sanctions of its own, but which can function as a means of keeping academic institutions up to scratch. As things are, institutions have more to gain from sweeping scandal under the rug of public ignorance than from the thorough investigation of accusations of misconduct. What is needed is a mechanism for compelling institutions to face up to their responsibilities on fraud. A central body that did no more than publish once a year a list of all investigations into misconduct by institutions in its purview, and which commented on their adequacy, would be a public service. But that is probably the most that can be done. □

Greek earthquake stirs controversy over claims for prediction method

London. An earthquake of 6.1 magnitude in southwestern Greece last week, which demolished two buildings and killed 20 people, has added fuel to a fierce scientific debate about a controversial method that claims to predict quakes by measuring increased electrical activity in the ground.

Panayotis Varotsos of the University of Athens, who developed the technique — known as VAN — accuses Greek seismologists of “playing with people’s lives” by denying the scientific validity of his method.

Using this method, he claims to have successfully predicted both the approximate timing and the location of the earthquake last week. He has now called on Greek seismologists to “drop their misguided opposition” to it.

Varotsos, who heads the solid state physics centre at the University of Athens, says that the Greek authorities were initially receptive to VAN when he correctly forecast a 6-magnitude earthquake close to Thessaloniki on 4 May and a 6.6-magnitude earthquake in northern Greece on 13 May.

The government, he claims, made a public declaration about the VAN method and promised to take countermeasures based on his predictions. But he says that the authorities reneged on their commitment when seismologists declared that the technique was scientifically unsound.

VAN is based on a discovery that Varotsos claims to have made in the late 1970s that materials under stress emit characteristic electrical signals. After publishing this research in what he describes as “many leading physics journals”, he decided to apply it

to earthquakes.

Monitoring stations exist at seven locations in Greece. Each consists of a network of electrode pairs designed to extract a clean electric field signal stripped of external ‘noise’ and ionospheric effects. Any unusual signal — such as a sudden burst of closely spaced peaks — is taken as a sign that stresses are building up, and that an earthquake will take place within a specified period, usually three to six weeks.

Varotsos says that, using the VAN method, he has correctly predicted the timing, magnitude and location of the three most recent earthquakes in Greece. He has been faxing information on the predicted timing and magnitude of each earthquake to 29 seismological institutes worldwide. “How many more people will have to die before this method is recognized as being correct.”

But many seismologists remain deeply sceptical, and say the method lacks the stamp of both scientific and statistical rigour. Xavier LePichon, of the Laboratoire de Géologie de l’Ecole Normale Supérieure in Paris, says Varotsos cannot claim success for VAN on a relatively limited number of observations.

David Booth of the British Geological Survey (but speaking in a personal capacity) says that VAN predictions on earthquake timings and locations, known as the ‘prediction window’, are “vague and loose”, and

Earthquake damage: how accurate are VAN predictions?

that earthquakes predicted by VAN have occurred later than expected and at different magnitudes. Even more significant, he says, is the fact that the connection between stresses in tectonic plates and electrical activity remains unproved.

A paper by four Greek seismologists that is being widely circulated says that Varotsos’ prediction of the Thessaloniki earthquake was five days too late and two magnitude units too large.

But Varotsos is standing firm, and says that his 13 May prediction was of an earthquake that lies in an area “where no earthquake has taken place for 1,000 years”.

Despite their doubts, not all seismologists dismiss VAN completely, and some are even beginning to investigate it further. Varotsos recently returned from a 10-day trip to Japan where the government (see left) has promised to invest heavily in VAN.

Last month, 40 scientists attended a closed two-day meeting on VAN organized jointly by Britain’s Royal Society and the International Council of Scientific Unions, where Varotsos and Seiya Uyeda, another leading VAN proponent who is based at both the University of Tokai and Texas A&M University, put their case for VAN.

At the end of the meeting, according to one delegate, up to half of the participants ended up “sitting on the fence”. The remainder were divided equally between supporters and sceptics. But the meeting is believed to have been boycotted by a number of Greek seismologists.

Sir James Lighthill, professor of mathematics at University College London, and the organizer of the meetings, says that its aim was to provide an impartial evaluation of VAN. The proceedings of the meeting are expected to be published by the end of the year.

Ehsan Masood

Japan jumps on board the VAN wagon

Tokyo. Japan is about to invest ¥250 million (US\$3 million) in the controversial VAN technique for predicting earthquakes (see above). The Japan Meteorological Agency (JMA) has included ¥200 million for VAN in a supplementary budget approved last month by the Diet to mitigate the effects of the Kobe earthquake.

The funds will be used to set up 20 detecting stations in the Kobe area to measure electric currents in the Earth near active faults. An additional seven will be established with wireless transmission of data for observing magnetic fields.

“Seiya Uyeda, a professor at both Tokai University and Texas A & M University in the United States, and a leading proponent of VAN, says he welcomes the JMA funding. He helped to arrange a visit by

JMA scientists to Greece in mid-March to investigate the technique.

In addition to JMA funding, the Ministry of Education, Science and Culture has allocated about ¥20 million in the supplementary budget to set up a VAN network at Hokkaido University. Uyeda’s group will help to establish the stations in Hokkaido and the private Tokai University, will provide Uyeda’s group with about ¥30 million to “revitalize” its VAN research.

Japanese earthquake researchers have in general welcomed the decision to put money into VAN, even though there is still considerable scepticism about the technique. Man-made electrical noise from train lines and other sources make it difficult to observe electrical activity of natural origin in Japan.

David Swinbanks

Heat gets turned up on climate research city

Boulder, Colorado. Proposed budget cuts at some of the world's leading atmospheric science laboratories could cause the loss of irreplaceable data on climate change, and set back understanding of global warming by several years, according to scientists working at the US laboratories concerned.

"There's a distinct difference between terminating long-term programmes and shutting down short-term programmes," says Dave Hofmann, acting director of the Climate Monitoring and Diagnostics Laboratory (CMDL) in Boulder, Colorado. "If you go for a year without collecting the long-term data, you lose it for ever," he says.

CMDL is one of six environmental research laboratories in Boulder run by the National Oceanic and Atmospheric Administration (NOAA). As part of the Department of Commerce, which Republicans in both houses of Congress would like to abolish, NOAA faces steep budget cuts — or even abolition (see *Nature* 375, 347; 1995).

As well as leading to gaps in the records of levels of greenhouse gas, ozone, aerosol and solar radiation kept by the laboratory, Congress's plans threaten to deal a blow to Boulder's economy, dominated by the concentration of government laboratories surrounding the University of Colorado.

The NOAA institutions under threat include the aeronomy laboratory, which has pioneered the study of ozone in the upper atmosphere, and the forecast systems and environmental technology laboratories, which each develop new technology for the National Weather Service and other users.

Like so many elements of the US research infrastructure, these laboratories have their roots firmly embedded in the Cold War: the aeronomy laboratory and the environmental technology laboratory — known until 1993 as the wave propagation laboratory — were set up in the 1950s because the US military wanted a better understanding of the upper atmosphere in order to improve its communications.

Department of Defense money still flows

into most laboratories. But their priorities have shifted to reflect concern about the global environment. "It's a good example of how science marshals itself to meet societal needs," says Fred Fehsenfeld, an atmospheric chemist at the aeronomy laboratory.

Yet this new mission has now become politically contentious. Dana Rohrabacher (Republican, California), chair of the House energy and environment subcommittee which oversees NOAA, said earlier this month that global warming was "unproven, at best, and liberal claptrap, at worst".

Scientists at Boulder are particularly concerned by a clause in an authorization bill for NOAA drafted by Rohrabacher that would constrain research to "seasonal" climate trends. The limitation, says Hofmann, is designed to put pressure on "anything associated with global warming".

The Rohrabacher language is unlikely to pass into law, but the pressure will remain. If research into long-term trends were to be halted, says Hofmann, most of the CMDL staff would leave at once.

In response to the threat to their future, the environmental laboratories have returned to first principles to explain what is known and not known about global warming and ozone depletion. "Scientists have tended to ignore the Rush Limbaugh types who say 'this is just a load of crap'", says Hofmann, referring to the influential radio talk-show host who argues that there is no problem of ozone depletion.

The laboratories have also been seeking political support by pointing out that their work does not necessarily lead to more government regulation. Research at Boulder on the use of methyl bromide as a fumigant in Californian agriculture, for example, indicated that it was being used safely, and supported the case against regulation.

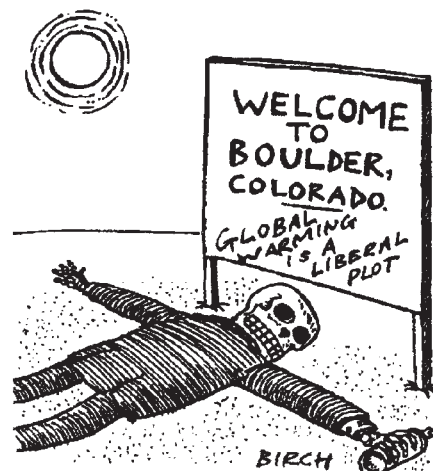
John Bates, a meteorologist at the climate diagnostics centre — another NOAA facility at Boulder — is about to publish research results on the stabilizing role of water vapour in the atmosphere

which he predicts will please those who claim that the dangers of global warming have been overstated. As a Democrat, he does not agree with their goals; but "it's important to see us as impartial scientists trying to understand nature better," he says.

As well as the research itself, pending cuts also threaten the comfortable lifestyle of the attractive and well-heeled city of Boulder, where three-fifths of the population have university degrees, and folks still leave their doors unlocked at night.

For example, 800 staff at a laboratory belonging to the National Institute of Standards and Technology — also part of the Commerce Department — face an uncertain future, with one Republican plan in Congress calling for outright closure.

At nearby Golden, the Department of Energy's 1,000-strong National Renewable Energy Laboratory works on programmes likely to lose at least half their funding. The University of Colorado, which received \$130



million in federal research last year, expects cuts in support from all its main funding agencies except the National Science Foundation (NSF). Fortunately for Boulder, the NSF is also the main source of finance for the National Center for Atmospheric Research, the city's largest employer — after the university — with 1,060 staff.

Cities such as Boulder normally receive solid support from congressmen and senators worried that, without them, their states will become intellectual and economic backwaters. But some staff at the laboratories say Colorado's two Republican senators have shown little interest, and they fear the city is about to suffer for its reputation as a hotbed of liberalism in this conservative state.

Others are more sanguine. Steve Clifford, director of the environmental technology laboratory, points out that the budget and government reform processes both have a long way to go in Washington. "What I'm interested in is the quality of the debate," he says. "If it's high enough, we'll come out okay."

Colin Macilwain

Security alert at French ion accelerator

Paris. The national heavy-ion accelerator at Caen in France (GANIL) has been shut down following the discovery of a failure in a safety system designed to prevent staff from entering a cyclotron while it is in operation, and thus being exposed to ionizing radiation.

The failure allowed access to one of GANIL's two accelerators — which was running at the time — for almost half an hour before being noticed fortuitously. It is thought to have been caused by software that had been recently modified.

No-one is thought to have entered the accelerator during this period, but

management at the reactor, which is jointly operated by the Centre National de la Recherche Scientifique and the Commissariat à l'Energie Atomique (Atomic Energy Commission), say that staff dosimeters have yet to be read.

The Department of Safety of Nuclear Installations, which made public the incident last week, rated it at 2 on the international scale from zero to 6 for nuclear accidents. It also ordered that the whole facility should be shut down pending proposals from its operators on how to prevent such an incident from happening again.

Declan Butler

UK panel warns of crisis in clinical research careers

London. The British government has been urged to set up a major review of academic careers in clinical medicine, in the light of uncertainties about research career prospects for people with medical qualifications resulting from recent reforms in the National Health Service (NHS).

The proposal comes in a report published last week by the House of Lords Select Committee on Science and Technology after a nine-month inquiry into the impact of the reforms on medical research.

The main focus of the report is the difficulties created for research supported by the NHS in the light of the government's introduction of an 'internal market' for the allocation of health resources. It points out that, in principle, the literal application of this philosophy could lead to "the end of curiosity-driven clinical research in the major university hospitals".

In general, however, the report has many complimentary things to say about the measures that have already been introduced to meet this threat. High among these measures was the announcement earlier this year that all forms of research spending by the NHS will be combined into a single, ring-fenced 'funding stream', and the extension of competitive funding for virtually all NHS-supported research projects, as proposed in a report published last year by a panel headed by Tony Culyer, professor of medical economics at the University of York (see *Nature* 371,275; 1994).

The Lords committee, whose members include a number of prominent former academics, says it feels it should take some of the credit for these moves, for which it has been pushing steadily since the government's reforms were first announced. At the same time, it pinpoints various aspects of the implementation of the Culyer proposals about which it continues to be concerned.

One urgent priority, it says, is to tackle the problem of clinical academic careers. "The evidence that an increasing number of doctors are choosing a career in clinical practice rather than academic medicine is very powerful," says the panel's chairman, Lord Walton of Detchant, formerly dean of medicine at the University of Newcastle.

Factors listed in the report as contributing to this situation include pressure on the funding of academic posts funded by the NHS, the impact of changes in the structure of medical education, uncertainty about whether academic achievements will be taken into account in locally agreed pay-scales, and whether parity will be maintained between NHS consultants and their colleagues in universities.

"We believe that the situation is becoming

so serious that it requires an investigation in its own rights," says Walton. He says that almost all the evidence submitted to his committee by a wide range of bodies, from university representatives to the British Medical Association, "points in the same direction", but he feels that so far the government "has not taken on board the crisis nature of the situation".

The committee also points out that there is growing concern in medical schools — particularly those in London, where the costs of patient care tend to be higher because of research overheads — about the difficulties caused by the financial rules under which local health authorities are now being required to operate.

"We have some evidence that managers in small regional hospitals are becoming reluctant to pay for tertiary refunds to centres of excellence, even with the higher levels of care [the latter] can provide," says Walton. "This is because their costs are high, while the internal market has created an environment in which local hospitals depend for their funding on the number of patients they treat."

More generally, the panel sees threats to the future of biomedical research from the 'bottom-line' mentality, which has inevitably come to dominate much thinking about the allocation of health-care funds allocated to doctors and local health authorities.

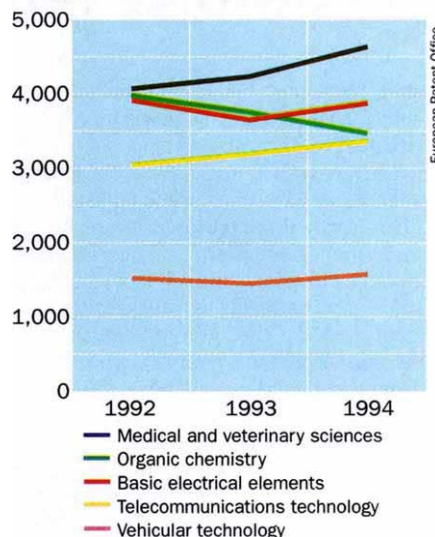
"We are concerned about the wall of accounting that has been created between clinicians and scientists, and researchers and administrators," says Walton. His committee expresses particular concern about the disappearance of the 'knock-for-knock' tradition, under which less costly items were shared by health authorities, universities and medical charities, and the increasing extent to which the latter two are being required to take on some of the basic costs associated with research that were formerly covered by the government.

The committee's report has been widely welcomed by both the academic and the biomedical community. Michael Powell, for example, of the Committee of Vice Chancellors and Principals (CVCP) — the body that represents British universities in their collective negotiations with government — says that his organization "welcomes all of the panel's recommendations".

Powell says that the CVCP is particularly pleased at the recommendation for an inquiry into clinical academic careers, a topic of increasing concern to university medical schools. But it is keen that this should not be purely an interdepartmental government committee, and should have an independent chairman.

David Dickson

European patent filings 1992-94



Life sciences gain favour in patents

Munich. After a gradual decline over the previous few years in the number of patent applications handled by the European Patent Office (EPO), they increased last year by nearly almost 5 per cent, to a total of 74,000.

Medical and veterinary technologies attracted the most applications. Indeed, the numbers of applications in this class rose faster than the average in 1994, by 9.5 per cent. Most of this increase came from the United States (39 per cent), France (22 per cent) and Japan (18 per cent).

In contrast, applications in organic chemistry — the class with the third-largest number of filings — fell significantly. Reduced filings from German, US and Italian companies accounted for most of this decline, which may reflect the switch in interest of the chemical industry from synthetic to biologically derived molecules and processes.

Nearly half of the applications originated in the EPO's 17 member states. Japanese companies were largely responsible for a rise in patent applications in the electrical and electronic industries, including telecommunications. A significant increase in patent applications for vehicle technologies reflected in particular a higher level of filings from German and US companies.

Paul Braendli, president of the EPO, who presented these figures in Munich last week, said that he welcomed the overall increase in the number of applications, but he expressed concern about the continued dominance of non-European applicants in high technology.

Acknowledging that the high cost of patenting through the EPO can put off small European companies (see *Nature* 375, 270; 1995), he announced that the office will abandon its proposal to increase fees by 3 per cent this year.

Alison Abbott

Economist promises aid to Italian research

Rome. "You can't remain both wealthy and stupid for more than one generation", says Romano Prodi, professor of economics at the University of Bologna — and a leading contender as Italy's next prime minister.

This is why Prodi puts education and science at the top of his political agenda. Most economic indicators, he says, show that Italy, particularly the north, is very wealthy, but urgent steps are needed to protect the next generation from the country's chaotic state.

Among other things, he says, this means considerably increased investment in science, and greater independence of universities from the state in order to bring together basic science and industrial needs.

Prodi, who is himself not attached to any

political party, leads the centre-left coalition which is currently fighting the conservative forces led by the former prime minister, Silvio Berlusconi, for power in Italy. Elections could be as early as the autumn.

Before his appointment at Bologna, Prodi headed the Istituto per la Ricostruzione Industriale (IRI), the major Italian public holding company, throughout the 1980s, and took up the position again in 1993 to oversee Italy's privatization programme. (He resigned when Berlusconi came to power last year.)

Prodi's background has given him strong views on the importance of science and research for industrial success. He points out that Italy is capable of world-class

science — as shown by the quality of its physicists — but says that the general quality of science is patchy.

He also points out that, although Italy is Europe's fourth biggest economy, its total investment in research — only 1.3 per cent of its gross national product — is not much more than half that of other major European countries. Increasing this figure is one of Prodi's priorities. "But there is no point in throwing money at the problem if you don't also change the quality of the organization," he says.

He is, for example, highly critical of the Italian habit, widely followed within the National Research Council (CNR), of distributing funds according to the "rain principle". Under this, everyone receives a small amount of funding, but there is no concentration of resources on outstanding projects. "The CNR is a place where small powers are balanced against each other," says Prodi. "It is not an agency where strong scientific choices are being made."

But he concedes that any change in attitude will be achieved only if it is accompanied by budget increases; the capping of CNR funds in recent years has left the organization with little room for manoeuvre, as most of its money goes on salaries rather than research programmes.

Prodi is critical of Italy's over-centralization of public structures, including both the CNR and universities. "Centralization in Italy happens in a bureaucratic way, without the intrinsic strengths that it could confer", he says, pointing out, for example, the ineffective way in which resources are distributed to the country's poorer regions.

One of his priorities is therefore to extend the degree of independence given to universities by a former research minister, Antonio Ruberti, in the late 1980s. But he also acknowledges that this has political implications.

He believes that he has a recipe for forcing change without provoking further ideological and economic objections. Prodi says he would encourage all universities to form agreements with industry — but would create special funds to support those in the south "to make up for their smaller opportunities to benefit from industrial support".

Prodi is aware of Italy's poor reputation as a partner in international projects. Whereas other countries tend to have agreed financing for the duration of the collaboration, Italian scientists are all too often embarrassed by having their own funding approved only one year at a time, usually two years in arrears. "Our French and German partners get tired and send the Italians to hell", he says. It would be both possible and imperative to create more continuity in international funding, he argues.

By profession, Prodi is an industrial

UK projects vie for millenium money

London. Projects designed to popularize science and protect the natural environment figure prominently on the short-list for a slice of £1.6 billion from National Lottery funds that the British government has reserved for "original and innovative schemes" to celebrate the beginning of the next century.

Proposals short-listed as potential recipients of Millennium funds — one

Jodrell Bank: hoping to popularize radioastronomy.

fifth of the lottery proceeds are being devoted to social projects — include a £110.8-million scheme for a high-technology campus in Birmingham, an £88-million plan to build a national science centre in a derelict quay area of Glasgow, and a £14-million exhibition centre, also in Scotland, on the work of Alfred Nobel and the winners of the prizes that he endowed.

There is also a £20-million proposal to rebuild the Jodrell Bank telescope (above) and construct a new visitors' centre. Sir Francis Graham-Smith, emeritus professor at Manchester University and former astronomer royal, says that the extra funds will enable Jodrell Bank to boost its twin role as an international centre for radioastronomy and promoter of public understanding of science.

The telescope is already due to be given a new surface to enhance its resolution, while a Cassegrain feed system will increase efficiency and allow interchangeability between frequencies. If Millennium funding is approved, says Graham-Smith, Jodrell Bank, which already caters for

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150,000 visitors every year, will also get a new control building where the public will be able to observe astronomers at work.

Other science-based projects to have reached the short-list of 83 proposals, each of which will now receive closer scrutiny from the nine Millennium Commissioners, include an electronic zoo in Bristol and £350,000 towards a centre in the Orkney islands dedicated to "a new approach to studying scientific concepts".

There were several ambitious science projects among the 467 projects not short-listed. One of those sent back for 'refining' was a £100-million plan for an art, science and technology centre around the national museums in South Kensington in London, known as 'Albertopolis'.

The eventual winners will be announced in September and will comprise 12 'flagship' projects and a number of smaller schemes. Each large project will receive between £10 million and £50 million, and will be expected to raise further funds from private sponsors. E.M.

David Parker/SPL

economist, and his desire for improved structures for basic science and education is accompanied by a strong belief that Italy must find ways of transferring knowledge of scientific advances to industry that exploit its unique cultural virtue of flexibility. "Our mentality is [one of] small groups, high energy," he says.

Most Italian industry is concentrated in small and medium-sized enterprises (5.2 million of industry's 6 million employees), which tend to be efficient but not innovative, with little interest in research. "You never find anyone on the board of an Italian company who comes from the research side", complains Prodi.

In addition, moving between public and private sectors in research is almost impossible; one cannot go to Fiat from the CNR and hope to return, because one will be viewed as a traitor, he says. Yet there is little career structure for researchers once they join industry.

Prodi has some specific proposals for closing the gap between basic research and industry. These include establishing the mutual recognition of career structures on the two sides, so that researchers can move freely between them without having to start at the bottom.

He also wants firmer control of Italy's new science park programme. A science park should be in the hands of a local consortium — including representatives of local industry and academics — to avoid problems that have arisen from, for example, professors "using the science parks simply as an extension of their own laboratories".

Even if Prodi were to succeed Lamberto Dini as prime minister, there is no guarantee that he would stay long enough in power to introduce the changes that he is proposing. But he is still taking science very seriously, both by visiting research institutes and consulting leading scientists. Many Italian scientists would like him to be able to do this from a position of political power.

Alison Abbott

India and Russia to re-build links

New Delhi. India and Russia last week announced an agreement to launch jointly up to 35 scientific projects at institutions in the two countries, and to create a common fund to meet the expenses of the scientists involved.

The agreement, which was signed in Moscow, represents a new start for Indo-Russian scientific cooperation, which was called off after Russia's decision to cancel a rocket deal with the Indian Space Research Organization two years ago.

A space probe designed to study X-rays from the sun and gamma radiation will be placed in Earth orbit in 1997. Other projects are primarily in areas of applied research.

K.S.Jayaraman

UK Labour Party seeks votes through pro-science image

London. Britain's Labour party, keen to project itself to voters as 'the party of the future', has set out to woo the scientific community with promises that science will be at the heart of the economic and social policy of any future Labour government.

John Battle, the party's science spokesman, last week promised, for example, that Labour would remedy the current lack of a strong, co-ordinated strategy for science across government, which he said resulted in initiatives by one arm of government (such as the Office of Science and Technology) often being undermined by the actions of another (such as the Department of Trade and Industry).

Battle also promised that the party would nail its colours firmly to the mast of technological change. Resurrecting the 1960s pledge of the then prime minister, Harold Wilson, to forge a new Britain in "the white heat of technological revolution", he said Labour would distance itself from "the negative and almost anti-science cultures of the late 1970s and 1980s".

But, in line with the party's general pre-election strategy, Battle declined to be drawn on details. In particular, while attacking an overall decline in government support for research and development, he refused to make any commitments to increased funding under a Labour government, arguing that "we cannot tell what we are going to inherit when we get into power".

Nor did he make any wholesale promises about re-nationalizing research facilities, preferring to focus his criticism — and suggested alternative strategies — on the fragmented way in which the government's privatization strategy has been carried out.

Battle was speaking at the inaugural meeting of a new group called Scientists for Labour, held at the London headquarters of the labour union Manufacturing Science Finance, whose members include university and government laboratory researchers.

Opening the meeting, Willie Russell, professor of biochemistry at the University of St Andrews, said that the spur for the creation of the group had been concern about "the collapse of scientific infrastructure and a crumbling of morale in the scientific community" in Britain.

Russell said that, for him, a key turning point had been the privatization of the

British Technology Group, a body originally established to plough back into laboratories the proceeds of exploiting publicly-funded discoveries, whose success had made it "an easy target for conservative ideologues".

Matthew Freeman, a genetics researcher with the Medical Research Council in Cambridge, and a member of the executive committee of the lobby group Save British Science, emphasized the need to protect the health and integrity of the science base.

While emphasizing that his group was political neutral, Freeman added: "We have reasonably high hopes that our views coincide with those that Labour are expressing, and that we can expect more from a Labour government than we have from the conservative government of the past 10 years."

A message from Tony Blair, the Labour leader, indicated that the party — which enjoys a substantial lead in opinion polls over the ruling conservatives under Prime Minister John Major — is keen to tap into such feelings among scientists. "Science is central to the shaping of our economic, social and political future," said Blair.

Battle reinforced this message by saying that, under Blair, Labour is committed "to help people to face up to technological change and make the world a better place," and that there is a need "to ensure that science is not feared or treated dismissively".

He was more cautious about the need for any changes in the substance of science policy under a Labour government. Following the upheavals resulting from the 1993 science white paper, for example, Battle said that the Labour Party has no desire to seek a new reshuffling of the research councils.

Indeed, he admitted to the scientists' group that "many of the ideas behind the white paper were good," endorsing, for example, the principles behind the technology foresight programme — while raising questions about the commitment with which its findings are likely to be implemented.

Battle also took issue with the 'short-termism' which, he claimed, remains "endemic to this government's attitude to science", quoting the problem of the increasing number of researchers employed on short-term contracts.

Keen to listen to scientists' complaints about the government, the Labour Party has organized six open meetings for the autumn in different cities in Britain. A lack of resources is likely to be high on the agenda. But even those who admit they are likely to be disappointed on this score suggest that the party's general attitude towards science is likely to weigh strongly. On that measure, Battle already appears to be scoring highly.

David Dickson



Battle: on the attack to defend research.

NASA scientists rapped over experiments with animals

San Diego. Scientists working at the US National Aeronautics and Space Administration (NASA)'s Ames Research Center in California have been authorized to resume experiments with animals following a two-month suspension because of deficiencies in the way the experiments were being managed.

NASA administrators say they are working on ways to correct the problems that led to the suspension in March of all animal projects at the space science facility. Meanwhile, the federal Office of the Inspector General is reported to be investigating allegations raised — including claims that an attempt was made to purge records of botched animal experiments.

NASA officials say that the suspension was costly and threatened to delay some space flight experiments, including a monkey project scheduled to be launched in August 1996 on the Russian Biosatellite. Animal experiments were resumed just in time to prevent projects from being switched to later space flights, said Kenneth A. Souza, assistant director for life sciences at Ames.

The shutdown of about 10 animal research projects, and the delay in the start of several others, came after the resignation in March of the facility's chief veterinarian, Sharon Vanderlip. Vanderlip complained that her efforts to ensure humane animal care and quality experiments had been thwarted by scientists and administrators.

"In my 15 years as a laboratory animal medicine veterinarian, I have never encountered the arrogance and blatant disregard for policies, regulations and animal welfare that I have witnessed at the NASA Ames Research Center," Vanderlip wrote in a letter of resignation to Daniel Goldin, NASA's administrator.

During her eight months in the job, Vanderlip said she found that animal experiments were bungled, that Ames did not have

an approved assurance letter filed with the federal Office of Protection from Research Risks, and that some scientists and administrators undermined the activities of the facility's animal care and use committee.

When she attempted to remedy these defects, Vanderlip said she was harassed and intimidated by some Ames scientists and administrators. "I believe in animal research," said Vanderlip in her letter to Goldin. But she added that she was "sickened by the lack of respect for the spirit of the guidelines which ensure animal welfare, validate scientific findings and reassure the public."

William E. Berry, acting director of space science, declined to comment on Vanderlip's letter, attributing some of the problems to administrative "restructuring". But a panel of four outside experts — three veterinarians and a physiologist — called to review Vanderlip's claims, issued a report on 3 April agreeing with many of her charges.

One panel member, Martin J. Fettman, a veterinarian pathologist at Colorado State University, wrote in the report: "Many federally required procedures for care and use of animals weren't developed or documented, the animal care and use committee wasn't functioning correctly, some animal experiments weren't properly conducted, and some records on faulty experiments were purged".

The review panel also found that an Oregon-based company, TEAM Support Services Inc., which Ames had commissioned to manage its animal care facility, had significant administrative problems. The \$1.8-million contract with TEAM Support is up for renewal. TEAM Support officials have declined to comment.

Souza at Ames says that the centre now has a "corrective plan" which is being implemented.

Rex Dalton

Fusion research cuts may mean closure for Princeton facility

Washington. Future prospects for the Princeton Plasma Physics Laboratory (PPPL) in New Jersey are looking bleak after a key congressional subcommittee voted to cut US spending on magnetic fusion research from \$360 million this year to \$230 million in 1996.

The House energy and water appropriations subcommittee, chaired by John Myers (Republican, Indiana), has not given any details of where cuts in fusion research should fall. But it has instructed the Department of Energy to work with Congress to map out a new programme that would fit the smaller budget.

PPPL currently spends \$65 million a year operating the Tokamak Fusion Test Reactor (TFTR), the main magnetic fusion experimental facility in the United States. It had hoped to start construction of a successor machine, the Tokamak Physics Experiment (TPX), next year, but laboratory officials recognizing that this was likely to be delayed for funding reasons, have been arguing instead for the continuation of TFTR to keep the laboratory alive.

Dale Meade, deputy director of PPPL, says that running costs for TFTR could be reduced to \$60 million and continued within the \$230-million budget, providing the United States cut back its other main fusion activity, namely its contribution to the design stage of the planned International Thermonuclear Experimental Reactor (ITER). "It depends how aggressively we continue with ITER," says Meade.

Meade says that a further three years of useful work could be done at TFTR, boosting the machine's power output from the 10.7 megawatts achieved last November to 20 MW, and gleaned more information about the behaviour of its mixed deuterium-tritium plasma fuel.

But influential House Republicans are ready to shut TFTR now. The House energy and environment subcommittee, chaired by Dana Rohrabacher has passed an authorizing bill that would do this, and Robert Walker, chair of the full House Science Committee, says the fusion programme should fund only ITER and a number of "small US programmes" to support it.

Earlier this year, a report from Congress's Office of Technology Assessment harshly criticized the lack of technical co-ordination between TPX and ITER, noting that TPX results would come too late to contribute to the ITER design. Democrat Congressman George Brown suggested that ITER might be delayed to use TPX results — a suggestion that has been angrily rejected by ITER management.

Colin Macilwain

AAAS seeks to fill policy advice vacuum

Washington. As the US Congress moves to shut its Office of Technology Assessment (OTA), the body that provides legislators with policy-related advice on scientific and technological issues, the American Association for the Advancement of Science (AAAS) is strengthening the assistance that it offers senators and congressmen.

The AAAS Center for Science, Technology and Congress last week held the first meeting of its advisory board, co-chaired by former congressmen John Brademas (Democrat, Indiana) and Bill Green (Republican, New York).

"The first thing I want to say is that we are not the OTA, and we don't have the OTA's \$22 million budget," Green told a congressional reception afterwards, attended by several influential legislators.

According to the AAAS, the centre will provide lawmakers and their staffs with "current, timely and nonpartisan information on science and technology issues". An AAAS spokesperson said its plans to develop the centre, which will take over work on science policy from the philanthropic Carnegie Endowment, pre-dated Congress' plans to kill the OTA. C. M.

ET search project in struggle for survival

Boston. After the demise of the National Aeronautics and Space Administration's (NASA's) Search for Extraterrestrial Intelligence (SETI) programme last year, and growing threats to efforts at Ohio State University (see *Nature* 374, 668; 1995), a project that has been run by the University of California, Berkeley, at Arecibo, Puerto Rico for the past three years may be the next victim of the US Congress' 1994 decision to cancel SETI funding.

"We're living on our residual NASA funds," says Stuart Bowyer, principal investigator for SERENDIP, a search that has been carried out at various locations since 1979. "That will keep us going for about six months, but then we'll have to stop unless we find some other means of support."

SERENDIP has been supported by NASA virtually from the beginning. "We know how to write grant proposals, but we don't know the first thing about fund-raising," says Dan Werthimer, programme manager for the project. "We're still struggling with that." A non-profit group, known as the Friends of SERENDIP, was formed in mid-1994 to handle contributions from private donors. But donations have been slow to arrive.

Ironically, the financial difficulties coincide with a major upgrade that will transform the present SERENDIP III system to the next generation, SERENDIP IV, creating a 40-fold increase in search capability. The new instrumentation is due to be installed by September, and will be able to inspect 174 million radio channels every 1.7 seconds, compared to the current 4.2 million channel system.

"We have enough money to get SERENDIP IV installed," says Werthimer. "It will be a shame to get that done and then have to shut down for lack of funds."

The frugality of the programme may be its salvation, as the Berkeley team needs only about \$65,000 a year to stay afloat. In contrast, the Phoenix project — part of the defunct NASA programme that was rescued by the non-profit SETI Institute and moved from Arecibo to the Parkes radio antenna in New South Wales, Australia — spends nearly 100 times as much.

Although SERENDIP is one of the most powerful SETI searches on the planet, it can get by on the cheap thanks to a neat design concept called 'piggybacking'. SERENDIP basically gets a free ride at Arecibo, operating alongside conventional radioastronomy observations without interfering with them.

While Phoenix scientists have to buy telescope time in order to pick and choose the stars in the sky they want to examine, SERENDIP is more like an eavesdropping device that listens in on radio 'conversations' other astronomers have already tuned into.

"It's an extremely cost-effective approach, because observing time at Arecibo is hard to come by and is also very expensive," says Mike Davis, project scientist at Arecibo. "They're one of the few programmes to get continuous telescope time here."

This is no small matter given that Arecibo is the largest radiotelescope in the world. By linking up to its 1000-foot diameter dish,

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Chance encounters? Arecibo radiotelescope by night.

SERENDIP has a sensitivity that other SETI programmes are hard-pressed to match.

The drawback is that the Berkeley group has no control over where the telescope is pointing. But Bowyer maintains that is not such a big disadvantage in SETI, as nobody knows where to look for extraterrestrial signals. "If we had complete control of the telescope and could do anything we wanted with it, we probably wouldn't do things much differently," adds Werthimer.

"Over time, SERENDIP covers a large fraction of the sky, although they survey it in a pseudo-random fashion," says Woody

Sullivan, a radioastronomer at the University of Washington, Seattle. "It's like a blindfolded guy mowing the lawn. Eventually he'll get most of it cut, save for a few tufts, even if he goes over it in a haphazard way."

Sky coverage is not the only objective, as searching for peculiar radio signals is really just half the game in SETI today. The other equally critical task is the steady improvement in technology that may ultimately lead to success. The SERENDIP team has been a leader on this front, providing the receiver used in the Ohio State search, as well as the general architecture for the spectrometer to be installed in BETA, the new SETI system designed by the Harvard physicist Paul Horowitz.

SERENDIP's biggest contribution, however, may be in introducing the piggybacking approach to radioastronomy, paving the way for similar efforts, either planned or in progress. A pulsar search led by Berkeley and the University of Cornell, which relies on this technique, is now under way at Arecibo, along with a spectral search for hydrogen that has been designed by Horowitz and scientists from Massachusetts Institute of Technology.

Horowitz has also developed a spectrometer with colleagues at Cornell that will soon be deployed in another piggyback-style pulsar search at Arecibo. "There are many other projects that could run in this fashion — basically searches that do not target specific places in the sky," Davis comments.

Steve Nadis

Big four dominate Australian research

Sydney. Just four of Australia's 34 universities account for nearly half of the research effort in the nation's higher education sector, according to statistics released last week.

The figures have been collected by the Australian Vice-Chancellors Committee, and represent the first detailed figures on university research funding in Australia — as well as a measure of the domination of the sector by a handful of institutions.

Of the \$A524 million (US\$390 million) spent on research by universities during 1993, the University of Melbourne accounted for 12.1 per cent, followed by the Universities of New South Wales (11.5 per cent), Sydney (11.1 per cent), and Queensland (10.5 per cent). These four older bodies were promptly dubbed the "sandstone club" by the media, a reference to the popular building material of colonial times.

Most of the remaining research funds were allocated to a clear second tier of

institutions. These include the Universities of Monash (7.9 per cent), Adelaide (6.2), Western Australia (6.1), and Flinders University in South Australia (3.1).

Judged in terms of publications alone (including book chapters, review articles and patents) Monash topped the rankings with 11.9 per cent of research output. In contrast, Sydney came first in the ranking of completed higher research degrees shows, with 344 completed masters and doctorate courses in 1992, and Melbourne came second with 304.

A comparison of the 1993 figures with those of the previous year show that research funding increased 11 per cent in 1993 with support from the private sector increasing 16 per cent to \$126 million. Frank Hambley, executive director of the vice-chancellors committee, said that the latter had been partly the result of a new co-operative research centre programme which had emphasized participating by industry.

Mark Lawson

European space science

SIR — As chairmen of the committees that prepared the Horizon 2000 and the Horizon 2000 Plus long-term programmes for space science within the European Space Agency (ESA), we wish to express our concern about the potential impact of recent proposals, in particular by the United Kingdom, significantly to reduce the funding of ESA's mandatory scientific programme.

Over the past ten years, European space science has reached a high level of achievement in many areas, despite a relatively low level of funding which is, for example, only one-fifth that of projects funded by the US National Aeronautics and Space Administration (NASA).

This has been made possible by a combination of well focused efforts, a carefully planned long-term programme based on the priorities of the scientific community, the timely development of new technologies and a continuous awareness of the need for efficiency and tight cost control.

A significant reduction in the ESA science budget, at a time when the Horizon 2000 programme has developed full momentum, would therefore have a devastating effect on European space science for a number of reasons.

First, the programme will suffer from a disastrous prolongation which is out of proportion to the budgetary decrease. A 10 to 20 per cent decrease, for example, would cause a much greater delay in the execution of the programme, and would lead to the cancellation of some projects on which funds have already been spent, as well as destroying the carefully structured balance between disciplines.

Furthermore, as the Horizon 2000 programme is fully under way, many individual missions are at an advanced stage of development. Prolonging such projects will inevitably lead to substantial increases in the costs incurred by industrial participants, which cannot be retrieved through cost-saving measures because contractual agreements are already in place.

Second, such delays will considerably increase the risk that delays in ESA missions will result in competitors being the first to obtain data of scientific interest. Europe would then return to its secondary role, having abandoned efforts to achieve pre-eminence which have been carefully nurtured over the past decade.

Furthermore, the time gap between successive missions in individual space science disciplines is already considerable. Both the Horizon 2000 and Horizon 2000 Plus programmes were felt to be the minimum needed to sustain the work of leading European space science laboratories and the associated technological developments in European industry.

Further delay will lead to frustration in the space science community, where many

scientists have built their careers on the promise of a stable and challenging European space science programme. In addition, creative young scientists and engineers will be discouraged from entering the space field, gravely damaging the long-term prospects for space science.

Third, although economies already achieved by the science programme mean that preparatory activities for Horizon 2000 Plus can be accommodated with present resources, achieving this has not been easy. Budgetary decreases now would mean having to sacrifice future developments in order to salvage as much of the present programme as possible, making the goals of Horizon 2000 Plus impossible to achieve.

For the same reason, existing hardware commitments will put a squeeze on funding to extend missions already in operation. Until now, economies and efficiency gains in the science programme have made it possible to pay for such extensions, sometimes far beyond the original planning period (for example with COS-B and IUE). The science return has in many cases been increased significantly for a relatively small additional investment.

In conclusion, we firmly believe that, without judging whether further economies can be made in ESA programmes in general, no further economies should be made in the resources currently available to the science programme. ESA is in considerable turmoil, and the science programme is one of its few stable elements, acting as the backbone of the whole agency. It is certainly important to seek further improvements in efficiency and cost-effectiveness of the science programme. But the potential savings should remain with the programme.

Ad hoc cuts applied by brute force, and without being based on solid evidence, to a programme in full operation would be utterly irresponsible and eventually counterproductive.

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Miracle or fake?

SIR — You report (*Nature* 374, 668; 1995) on the case of a figurine of the Virgin Mary that was widely claimed in the Italian media to be weeping "tears of blood".

Is this a miracle? As a scientist and

agnostic — despite a Catholic education — I am obviously sceptical. But, as an Italian citizen, I am surprised that a judge, who is an official of the Italian state, should have decided to investigate the authenticity of the so-called miracle, and that he should do so using apparently tough methods.

The judge has "arrested" the figure, even though it had been brought — and not even sold — to a local church by its owner. He is now attempting not only to submit the tears to chemical analysis, but also to apply DNA tests to the owner of the figurine and his family. As you report, such analysis had already been carried out by scientists at the Policlinico A. Gemelli, a Roman Catholic medical school in Rome, at the request of the Catholic Church. These scientists have already concluded that the tears do indeed contain human blood. But whatever the final explanation, I feel that the only authority that should be investigating the affair is the Catholic Church, with reference to scientific institutions or other consultants if it so chooses.

It is then for the church to decide whether the figure is a 'miracle' or a 'fake' — and for others to accept or reject its conclusions. Bureaucrats and scientists should resist the temptation to impose a reverse inquisition, which would not be compatible with either our constitution or the relationship between the Italian state and the Vatican.

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Citation analysis

SIR — Calza and Garbiza (*Nature* 374, 492; 1995) state: "Computerized analyses of scientific publications allow immediate comparison of applicants by objective parameters", I should like to quote Sydney Brenner (*Current Biology* 5, 568; 1995): "Before we develop a pseudo-science of citation analysis, we should remind ourselves that what matters absolutely is the scientific content of a paper and that nothing will substitute for either knowing or reading it. We should also recognise that citation often tells us more about sociology of science than about science itself." The assumption that fairness in competitions for university positions can be assured by the proposed allegedly objective criteria is simply ridiculous.

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Radioactive waste disposal

SIR — I have been following the debate on radioactive waste disposal for some time in *Nature* and elsewhere¹. I accept that encasement and secure entombment is a viable means of disposing of high-level radioactive wastes, but the vexing problem of large volumes of medium- and low-level wastes remains. Deep slurry fracture injection into ancient geological sedimentary formations may represent an economic and socially acceptable means of executing this task.

For several years, we have been injecting large volumes of contaminated sand into porous, permeable, friable sandstone strata of Cretaceous age (Mannville Group, >10⁶ million years BP) in Alberta and Saskatchewan. The sand is a waste product of heavy oil production² and it is injected under pressures exceeding the overburden weight using an aqueous slurry, thereby fracturing it into the permeable sand. The aqueous phase dissipates by Darcian flow, and the sand remains in the near vicinity of the wellbore permanently entombed and immobile under the high stresses arising from the overburden weight.

In one case, still active, in excess of 12,000 m³ of sand has been injected into a depleted oil formation over a six-month period. Monitoring using adjacent wells and surface deformation field inversions demonstrated convincingly that the solid wastes are contained locally and that pressures dissipate rapidly. Injection depth is 400 metres, and environmental security is provided by a thick smectitic shale overburden (> 200 metres), flat-lying stratigraphy, absence of tectonic features and great lateral extent of the target strata. The interstitial water in these strata is older than 10⁷ years, and flow velocities are of the order of centimetres per year. Also, there are clay minerals dispersed in the sandstones, as well as in the adjacent shales, which should readily adsorb any polyvalent metal cations that leach from the wastes. We believe it is reasonable to speak in terms of million-year security for slurry fracture injection, given the proper geological conditions. Later this year, disposal of potash processing slimes (inert mineral matter) using the same approach will be undertaken with a comprehensive monitoring strategy.

Low- and medium-level, low-solubility solid radioactive wastes are excellent candidates for this type of disposal, and enhanced security could be achieved by adding a preponderance of shale fragments to the injected slurry, to provide local adsorption sites. Thus, given the right geological site (and many such sites exist), a granular solid waste can be prepared and injected with current petroleum industry technology at depths sufficient to entomb the wastes permanently, allowing

the radioactive species to decay slowly. Our recent success with this leads us to suggest that it be considered for such materials, removing a barrier for the continued peaceful use of nuclear energy.

Maurice B. Dusseault

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1. *Nature* **375**, 91–92 (1995).
2. Dusseault, M. B., Bilak, R. A., Bruno, M. S. Rothenburg, L. 1995. *Disposal of Granular Solid Wastes in the Western Canadian Sedimentary Basin by Slurry Fracture Injection*. Proceedings, Deep Well Injection Symposium, Berkeley, California (in the press).

Mannerly research

SIR — No one would disagree with the basic sentiment expressed in your leading article (*Nature* **375**, 4; 1995) that personal disputes should not interfere with scientific publishing, but in the particular case referred to we believe there may be misperceptions about what is considered as “good manners” in scientific research.

Six years ago, Professor Kenneth S. Kendler of Virginia Commonwealth University (VCU), together with staff from the Department of Mental Health at the Queen's University of Belfast and the Health Research Board in Dublin, began a study of the genetic epidemiology of schizophrenia in Ireland. Given the collaborative nature of this project, firm agreements were established at the outset about ownership of the data collected and authorship of any future publications. It would have been impossible to undertake this type of project without this explicit understanding and mutual trust. Throughout this study, we, the authors of this letter, have been fully involved in discussions with Kendler about future directions and possible publications relating to the project. One of us was involved in a site visit by members of the US National Institute for Mental Health to VCU which led to an extension of the funding of the study.

Dr Scott R. Diehl was an important part of this collaboration until two years ago when he left the laboratory at VCU. After his departure we were aware of the continuing dispute between Diehl and Kendler. However, during this time Diehl has had no communication with us either about his role in the project or the use of the data collected. We were therefore unaware of recent publication based on this subject until a few days before it appeared in *Nature Genetics*. We find it unacceptable that this publication did not properly acknowledge the other institutions involved or the collaborative nature of the study. It is unclear from the published paper whether Diehl has in fact taken data from VCU without the agree-

ment of the collaborating partners.

By contrast, Diehl was kept aware of the continuing work on the chromosome 6p region in the laboratory at VCU and was given a copy of the manuscript that was to be submitted and in which Diehl's role in the original finding was acknowledged.

It is therefore difficult for us to accept that “no one behaved wrongly”; indeed we believe that the issues involved go far beyond those of “priority” in scientific publications.

Finally, borrowing on your metaphor, one member of the original partnership group did publish with the author list chopped in half. We wonder what Solomon's judgement would have been in this case.

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Dundee did well

SIR — Ehsan Masood (*Nature* **375**, 96; 1995) correctly pointed out that the University of Leicester's biochemistry department overtook Dundee in terms of research income per full-time academic according to the CVCP/UFC Management Statistics for 1993–94. Despite some anomalies in the calculations (for example establishing an MRC unit removed a considerable source of income from our return), Dundee came a close second, ahead of both Oxford and Imperial College and not, as the article implies, somewhere among the also rans.

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Earlier than that

SIR — John Maddox (*Nature* **374**, 759; 1995) is incorrect in stating, with regard to the success of Einstein's theory of general relativity, that: “The bending of light near the limb of the Sun at the solar eclipse of 1919 was the first validation . . . The precession of the perihelion of Mercury came later.”

According to Steven Weinberg (*Gravitation and Cosmology*, page 12, Wiley, New York, 1972), the precession of the perihelion of Mercury was discovered by LeVerrier even before LeVerrier predicted the existence of Neptune. The perihelion precession was confirmed in 1882 by Simon Newcomb.

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Preserving embryos

SIR — The question of human embryo cryopreservation is being discussed again in the scientific journals^{1,2} as well as in quality newspapers³. The discussion arose from a paper by Dulloust *et al.*⁴ that claimed that in a long-term study comparing the development of cryopreserved and control embryos of mice, the researcher observed "significant differences" in morphological and behavioural features. They also advised researchers to make "a more limited use of this [cryopreservation] technique in clinical practice until clear conclusions about its effects on human embryos can be drawn". This led to comments in the French Parliament and the British House of Commons⁵ and caused some concern among researchers. The authors of the study have, however, issued a statement to the effect that their "significant results" are similar to those usually observed between two different lines of mice. In short "the significant results" have no significance.

The question of the effect of cryopreservation on human children naturally concerns us. Fortunately, there is no society, civilized or otherwise, that would allow a statistically designed experiment to be carried out in this area. No firm conclusions can, therefore, be reached about the effect of cryopreservation of embryos or sperm. This does not, however, mean that no "working" conclusions can be drawn about the development of children born by medically assisted procreation (PAM). In this situation there is a well-trodden tradition of using observational studies. We can design such studies to compare the development of some abilities in PAM children with a matched group of other children. Indeed, with other French researchers⁶, I carried out such research in France. Its aim was to see if the development of some behavioural traits differs in PAM and other children and to carry out appropriate genetic analyses. We collected data on 103 children born by artificial insemination by donors (frozen sperm) and on a matched sample of 103 children. Regrettably, the analysis of the data has been delayed and I shall report the results as soon as preliminary analyses have been made. I can, however, say that I noticed no material effects.

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1. *Science* **267**, 618–619 (1995).

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Greenhouse gases

SIR — There are no free lunches; nuclear power is as clear an example of that as the greenhouse gases resulting from fossil fuel combustion. Nuclear power carries with it the myth of limitless 'clean energy'. In order to judge the value of a procedure, the history of the practice and results of its use must be considered. That of nuclear power is fraught with errors and misuses, not the least of which are the production of nuclear weapons and accidents such as that near Chernobyl. One possible approach to replacing fossil fuel combustion is "making nuclear power usable again" (*Nature* **375** 91; 1995). There are many other ways of generating power and, more importantly, of reducing the amounts we use now.

Very few methods of generating electricity result in zero harm to ourselves and other natural systems. Hydroelectric dams kill anadromous fish and the making of solar energy panels requires the use of toxic chemicals. Nuclear power results in very dangerous waste products in liquid and solid form. Governments and private companies involved in developing this power-generating technique have a history of deceiving communities near the power plants, sources of uranium and sites of waste disposal. There is no reason to think that if the scale of nuclear power were to dramatically increase, these agencies would suddenly act in a more responsible manner; the converse is probably closer to the truth.

The solution to greenhouse gas production certainly lies in reducing reliance on the sources of this global pollutant. However, if the first alternative advocated is the re-adoption of nuclear power, despite its tattered history, then the scientific and technological communities will have certainly renounced their responsibility to act ethically. Reduction of rates of consumption and production are at the heart of a rational and sustainable way of living in our finite world. Even if this means a change in our standard of living, or the amount of high-tech science we can do, we must embrace it nonetheless, rather than grasping at the nearest available replacement to petroleum regardless of consequence.

Fraser Shilling

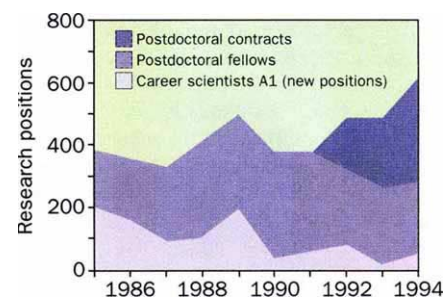
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Spanish science

SIR — Human capital investment in Spain is an explicit priority in science policy, since the number of scientists and engineers in research and development is far from European Union standards. However, in spite of the Spanish public

efforts in the training of young scientists (see *Nature* **360**, 502; 1992), little has been achieved in offering them stable positions. From 1990 on, the central and regional administrations have invested more than US\$500 million in support of a yearly average of 8,000 graduate and 2,000 postdoctoral fellowships.

As the number of postdoctoral trainees has increased in both quantity and quality, the continuous shrinkage in research jobs has frustrated the expectations of the young scientists. To cope provisionally with this widening gap, the government decided in 1992 to provide three-year contracts to young scientists, but the problem has now worsened. Take, for instance, the case of the Spanish scientific research council (CSIC), the leading Spanish research institution in which one-third of those researchers at present work: an



Job opportunities for young scientists in the Spanish scientific research council (CSIC). Data from CSIC's annual reports.

increasing number (nearly 600) of total postdoctoral fellowships and contracts is faced with a frozen number of new staff positions (an average of 45 annually in the past three years). Besides this, very few of these scientists have a real option to enter academic positions because of the secular endogamy in the universities. On the other hand, the few job opportunities in industry are in general adapted to less qualified candidates.

The general dissatisfaction with this situation is demonstrated in an open letter to the heads of the Spanish state and government, which is receiving massive support among the research staff in the CSIC. They are demanding a two-year extension of current short-term research contracts, after evaluation of scientific performance, and the prudent transformation of these contracts based on the tenure-track model. Thus, after years of hesitation, science policy authorities are confronted once again with the need to bridge the gap between science training and scientists employment.

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Elastic properties of DNA revealed

A neat technique originally devised for straightening molecules of DNA turns out to be an effective way of measuring the elastic properties of the molecule.

WITH the passage of time, virtually every aspect of the properties of the archetypal DNA molecule will have been investigated. For such an important structure, nothing less can be expected. But there may be particular interest in a way of measuring the mechanical strength of duplex DNA molecules that has now come to light. At this stage, the numerical result may be less important than the neat method by which it has been obtained, and which may have more general usefulness in the investigation of single molecules.

How can such a measurement be accomplished? The first need is evidently a way of anchoring a single molecule, a question amply explored in the past few years because of the interest in the uses of tethered oligonucleotide fragments of defined and different structure as means of capturing complementary polynucleotides from solution. It remains to be seen whether such a technique will offer advantages in the rapid sequencing of DNA, but it cannot be long before "DNA on a chip" is used in diagnostic kits of various kinds. (The purchase by Glaxo of the company called Affymax refers.)

But even when a molecule of DNA has been anchored to a solid surface, it is far from obvious how to apply to it a steadily increasing force that is not so great that it pulls the molecule apart immediately. The old school-laboratory experiment for the measurement of Young's Modulus, in which weights are added in succession to the bottom of wire hung from, say, a ceiling, is the model. A group in Paris has found an ingenious solution for molecules of DNA: anchor a molecule at both ends to a solid surface, immerse the whole in an aqueous solution and then withdraw the liquid slowly, so that the middle section of the DNA molecule is pulled away from its anchorages by the retreating meniscus of the liquid.

The advantage of this arrangement is that the force exerted on the molecule is self-limiting. As the meniscus retreats, it feeds out extra DNA as the reel on a fishing-rod releases extra line. At least in the early stages of the process, the molecule is unlikely to snap physically. The designers of this arrangement are D. Bensimon, A.J. Simon and V. Croquette from the Ecole Normale Supérieure and A. Bensimon from the Institut Pasteur (*Phys. Rev. Letts* **74**, 4754-4757; 1995). A similar procedure, called "molecular combing", is applied to DNA molecules anchored at one end only to pull them straight and align them in

one direction on a solid surface.

In the established tradition of most studies of the physical properties of DNA, Bensimon and colleagues have worked with the DNA of bacteriophage λ . There are several ways of anchoring DNA molecules to surfaces, which must be coated in advance with suitable anchorage material. The essential requirement is that DNA molecules should be able to bind only at their ends. To make them visible, the molecules are stained with a fluorescent dye.

Even the control experiments the Paris group describes are telling. In combing DNA molecules anchored only at one end, it turns out that the average length of a λ -molecule amounts to 24 μm when the surface carries a hydrophobic coating (such as a silane), but is about two-thirds as much when the surface is hydrophilic. The inference is that the retreating meniscus can more easily stretch an anchored DNA molecule when the molecule has no particular affinity for the underlying surface than when the affinity for the surface is strong, as it must be when the surface is hydrophilic. The actual numbers show that λ -DNA can be extended by at least 50 per cent without snapping.

The stretching of doubly anchored λ -DNA by a retreating meniscus is more revealing. What then seems to happen is that, after the meniscus has retreated beyond both anchorage points, the length of the molecular tether to each anchorage point is extended in a direction perpendicular to the retreating interface. Only when the length of the DNA within the retreating liquid is equal to the distance between the two legs do unexpected things happen.

Then, it is not difficult to imagine that it will no longer be possible for the two legs of the molecule to be extended in a direction perpendicular to the meniscus, and that the tension along the length of the DNA molecule will at this stage be steadily increased. After the meniscus has left the whole molecule high and dry, the result is a shape in which two parallel legs are joined by a roughly semi-circular shape, making an overall shape not very different from a vertical cross-section through an old-fashioned test-tube.

According to Bensimon *et al.*, the remarkable feature of these shapes is that (for the same surface coverings) they are identical except for a scale factor determined by the distance between the two parallel legs. Another, vividly apparent from the photographs accompanying their

article, is that the DNA molecule may sometimes snap and that, if it does, the break will appear at the curved end of the loop and will be made apparent by two dangling lines of fluorescence conveniently aligned in the direction of the retreating meniscus.

From that phenomenon, it is possible to estimate the degree to which DNA can be stretched without breaking. The argument goes like this: in a curve-ended loop of DNA with two dangling pieces of DNA, the width of the gap presumably represents the length of the fully-stretched DNA, while the two dangling pieces are the unstretched length of the same DNA segment. By using molecular combing as a way of calibrating these measurements, Bensimon *et al.* conclude that a DNA molecule can be stretched to roughly twice its native length before snapping.

By any standards, that estimate (the average of 25 measurements) is surprisingly high. How the normal duplex configuration could allow such great extension are not easily pinned down. Presumably an untwisting of the helix over relatively short lengths of an entire molecular would be energetically more favourable than a separation of the two antiparallel strands, but the point would bear investigation. Meanwhile, it also emerges that the shape left by a doubly anchored molecule from which the meniscus has retreated can be calculated explicitly, leading to formal expressions for the shape of the fluorescent molecules left behind by a retreating meniscus that differ only in the value of a single parameter. For a particular value of this constant, the blunt end of an unbroken doubly-anchored molecule is accurately a semicircle. In principle, it should be possible to turn this argument around, and to calculate from the shape of a doubly anchored molecule (whether or not it snaps) the controlling elements of any particular measurement, of which the chief seems to be the affinity of stranded DNA for the underlying surface.

The numbers have some interest. Young's modulus, for what it is worth, turns out to be $1.1 \times 10^8 \text{ N m}^{-2}$. But that must be a composite figure; with such a structure, Young's modulus should vary with the strain. The force needed to break a DNA molecule is estimated at 480 pico-Newtons, but with an error of 16 per cent. (From that, it should readily be apparent that a 1 mm thread of DNA would support a weight of 120 kilograms.)

John Maddox

Yielding under the strain

C. Weissmann

THE nature of the agent responsible for transmissible spongiform encephalopathies such as scrapie in sheep, mad cow disease or Creutzfeldt–Jakob disease in man has been the subject of impassioned controversy. The agent (known as prion) is thought to be a modified, pathogenic form of a constitutive host protein which multiplies by converting the normal form into a likeness of itself¹. This hypothesis has been cast in precise molecular terms² with the isolation of the host protein, PrP^C, and its modified form, PrP^{Sc}, both of which are encoded by the same gene³ and are believed to be conformational isomers. Support has steadily accumulated for this ‘protein only’ hypothesis, but the existence of many different strains of prions that can be propagated in mice homozygous for the single-copy PrP gene⁴ raises the question of just how prion strains with different phenotypes can be encoded by a single polypeptide. Now, on page 698 of this issue⁵, Bessen *et al.* report that when PrP^C from uninfected cells is incubated with PrP^{Sc} derived from mouse brains infected with different prion strains, it is converted into PrP^{Sc}; moreover, the biochemical features of this PrP^{Sc} are the same as those of the PrP^{Sc} used as ‘template’, which suggests that strain differences arise from different conformations of PrP^{Sc}.

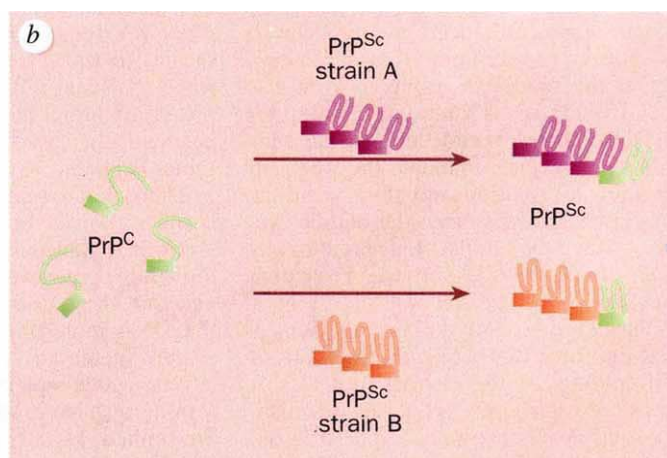
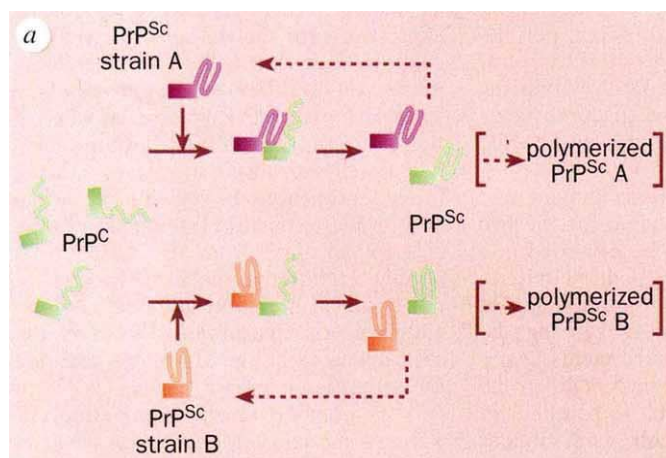
A wealth of evidence supports the ‘protein only’ hypothesis and incriminates the infectious agent as a deviant form of PrP. Copurification experiments have demonstrated the link between infectivity

and PrP^{Sc} (ref. 2). A scrapie-specific nucleic acid has never been found², arguing against the virus and virino⁶ hypotheses. Because the ratio of infectious units to PrP^{Sc} molecules is only about 1:100,000 (ref. 7), the structure of the PrP molecule actually associated with infectivity is hard to infer. For this reason, and because specific infectivity can vary considerably, the PrP species responsible for infectivity is for the time being better designated as PrP* (ref. 7), which may or may not be identical with PrP^{Sc}, the major species characterized chemically and physicochemically. If it is identical, the low specific activity could be due to a low efficiency of infection or to the infectious unit being an aggregate of a large number of PrP^{Sc} molecules.

Genetic evidence linking PrP to prion diseases is compelling. Ablation of the PrP gene renders mice resistant to experimental scrapie and precludes replication of the infectious agent; reintroduction of PrP transgenes restores susceptibility to scrapie⁸. The so-called species barrier, which hinders prion transmission from one host species to another, is overcome by introducing PrP transgenes from the prion donor to the recipient^{8,9}. Remarkably, all familial cases of transmissible spongiform encephalopathy in man are probably linked to one of many possible mutations in the PrP gene¹⁰; these mutations are thought to facilitate occasional spontaneous conversion of PrP^C to PrP*, which is then followed by the autocatalytic process.

PrP^C and PrP^{Sc} are chemically indistinguishable¹¹ but differ in their secondary structure and physicochemical properties. PrP^C has a very low content and PrP^{Sc} a high content of β -pleated sheet structure¹². Under conditions where PrP^C is readily degraded by proteinase K, PrP^{Sc} is partially resistant: about 60 amino-terminal residues are cleaved off and the remainder of the molecule is quite stable. PrP^{Sc}, unlike PrP^C, readily forms aggregates and gives rise to the fibrils and amyloid plaques pathognomonic for prion diseases.

Conversion of PrP^C to PrP^{Sc} in scrapie-infected cells is a late post-translational process, occurring after PrP^C has reached its normal extracellular location or later¹³. Spontaneous conversion of PrP^C to PrP^{Sc} (or PrP*) is obviously — and luckily — an extremely rare event. Why is this so, and how does scrapie infection promote conversion? The ‘refolding model’ (a in the figure) proposes that conversion requires PrP^C to be unfolded to some extent and to be refolded under the influence of a PrP^{Sc} molecule, a process that might have to overcome a high activation energy barrier and require a chaperone and an energy source². The ‘nucleation model’ (b in the figure) proposes that in the presence of a ‘seed’ of (polymeric) PrP^{Sc}, accretion and subsequent rearrangement of PrP^C occurs^{14,15}; the trapping of PrP by an essentially irreversible aggregation reaction would drive the bulk conversion process. The proposed process is akin to the assembly of, for example, (protease-sensitive) flagellin to (protease-resistant) flagellar filaments¹⁶. Interestingly, the same flagellin molecule can assemble into two types of flagella depending on the provenance of the seed¹⁷,



Refolding and nucleation models for the conversion of PrP^C to PrP^{Sc} in different prion strains. a, Refolding model (based on ref. 2). PrP^C has a stable conformation: for conversion, PrP^C must be partially unfolded and refolded under the direction of PrP^{Sc}; this may require a chaperonin and an energy source. PrP^{Sc} may subsequently aggregate to fibrils, rods or amyloid under appropriate conditions. b, Nucleation model (based on refs 14,15). PrP^C has a partly flexible conformation: after binding (perhaps weakly) to the PrP^{Sc} polymer, a conformational rearrangement takes place which ‘adapts’ the incoming PrP molecule to the polymer. The reaction may be driven in part by the deposition of monomers into

insoluble polymer. The important difference between the two models lies in the assumption that in the unfolding model, PrP^{Sc} conformations are inherent, stable properties of PrP monomers or dimers, and that protease-resistant PrP^{Sc} can occur in a non-aggregated, soluble form², whereas the nucleation model presumes that the properties of PrP^{Sc} are not a stable property of a PrP monomer or dimer but are acquired within the framework of the polymer. PrP^C molecules undergoing conversion are shown in green, with the box representing the stable moiety of PrP and the tail the conformationally variable moiety; purple and orange represent conformations specific for two strains of PrP^{Sc}.

providing an analogy for conformationally determined prion-strain specificity.

Whatever the nature of the conversion process, an important experimental advance¹⁸ has been the demonstration that incubation of ³⁵S-labelled biosynthetic hamster PrP^C with about 50-fold excess of PrP^{Sc} from scrapie-infected hamster brain results in the conversion of some labelled protein into a state that is partly resistant to proteinase K, like hamster PrP^{Sc}. To achieve this conversion, ³⁵S-labelled PrP^C and PrP^{Sc} were partly denatured with 3 M guanidinium chloride before incubation in a lower, critical concentration of the denaturant. Because of the denaturation step, the experiment is compatible with both the refolding and the nucleation models. The large amount of infectious agent associated with the PrP^{Sc} added, the modest extent of conversion and the imprecision of the infectivity assay currently preclude attempts to search for an increase in infectivity. The specificity of the reaction was shown by the fact that murine PrP^C was converted by murine PrP^{Sc} but not by hamster PrP^{Sc}, as would be expected from the species barrier against *in vivo* transmission from hamster to mouse; however, there was conversion in the converse reaction, which might reflect a less stringent barrier from mouse to hamster¹⁹.

In their *in vitro* investigation of the propagation of strain-specific properties of scrapie prion protein, Bessen *et al.*⁵ have taken advantage of the finding that two hamster-adapted scrapie strains, HY and DY, give rise to PrP^{Sc} molecules that are cleaved to products of different lengths by proteinase K (ref. 20). In the system described above, ³⁵S-labelled hamster PrP^C incubated with DY PrP^{Sc} gave a radioactive proteolytic product which was shorter by about 1K than that

obtained after incubation with HY PrP^{Sc}, the same difference as that found for the protease-resistant moieties of natural PrP^{Sc} molecules associated with the two strains. Although no chemical analysis of the ³⁵S-labelled PrP^{Sc} was done, it was assumed that the chemical structure of the molecule was unchanged and that the strain-specific susceptibility to digestion by proteinase K must be due to differences

in conformation. These remarkable results, if confirmed and extended to show that conversion can also generate infectivity, will finally vindicate the 'protein only' model, allow structural characterization of the prion and solve the strain problem.

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ATMOSPHERIC CHEMISTRY

Dynamics of the carbon cycle

Martin Heimann

HARDLY any observational record has had as much impact on those concerned with the present and future climate of the Earth as the famous CO₂ concentration record measured at Mauna Loa Observatory on Hawaii. On page 666 of this issue¹, Keeling *et al.* provide the latest update of the record, supplemented by observations from the South Pole. Both sets of data now cover more than 35 years. Furthermore, Keeling *et al.* present a parallel time series of measurements of the ¹³C/¹²C stable carbon isotope composition of atmospheric CO₂ over the past 15 years.

Atmospheric CO₂ is relatively chemically inert, and is controlled almost entirely by surface source and sink processes. Variations in these processes occurring on timescales longer than typical atmospheric mixing times (that is, more than 1–2 years) are reflected in global-scale fluctuations in concentration, and show up at stations monitoring background concentration. Indeed, close inspection of the long, homogeneous records observed and analysed by Keeling *et al.* reveals an amazing wealth of information, both on the direct anthropogenic effects and on climate-induced perturbations of the natural carbon cycle.

Clearly, the major part of the concentration rise of approximately 45 parts per million since the beginning of the record is a direct consequence of the anthropogenic emissions from fossil fuels and changes in land use (deforestation for instance). This is illustrated by a remarkable correlation between the cumulative industrial CO₂ emissions and the atmospheric inventory, suggesting that over the past 35 years an almost constant fraction (about 56 per cent) of the fossil-fuel-derived CO₂ remained airborne. The remainder must have been absorbed by the terrestrial biosphere and the oceans. The relative roles of these two sinks of anthropogenic CO₂ in affecting the atmospheric CO₂ budget have been highly controversial; even now the budget can only be quantified with considerable margins of error².

Calculation of the 'CO₂ anomaly', which is done by subtracting the seasonal

cycle and the industrial trend from the atmospheric record, reveals small but significant deviations which cannot be explained by temporal changes in anthropogenic emissions. Variations on two timescales are evident: decadal fluctuations and variations on the El Niño/Southern Oscillation (ENSO) timescale of 3–5 years. These variations are clearly correlated to climate records, such as the Southern Oscillation index or global surface temperature^{3–5}. Identifying their exact cause, however, remains a big challenge. But given that terrestrial and oceanic source processes are at work, which is the main source of these variations?

This is where information on the ¹³C/¹²C ratio comes in. The ratio in terrestrial CO₂ (including that from fossil fuels) is depleted by about 18 parts per thousand, whereas CO₂ of oceanic origin has an isotopic composition close to that of atmospheric CO₂. Measurements of the ¹³C/¹²C ratio in the atmosphere may thus be used to estimate the relative contributions of terrestrial and oceanic source and sink processes. Such calculations are not without pitfalls, however, in that various fractionation processes occurring during gas exchange between the terrestrial biosphere and the ocean surface are only poorly understood. An additional complication arises from the isotopically light CO₂ from fossil fuels, which introduces a transient isotopic perturbation into the carbon system. In consequence, isotopic disequilibria between the various reservoirs have to be taken into account in the calculations⁶. In their analysis, Keeling *et al.* use a robust reservoir model of the global carbon cycle to calculate these isotopic disequilibrium terms.

On the ENSO timescale, changes in the isotopic disequilibria are less of a problem and the isotopic record can be used to determine anomalous fluxes (deviations from the longer-term trend). The fluctuations in these anomalous biospheric and oceanic fluxes, as deduced by Keeling *et al.*¹ from the CO₂ and the ¹³C/¹²C record, are impressive and reveal a dynamic pic-

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ture of the global carbon cycle. During strong ENSO events (as in 1983 or 1987) the ocean represents a significant anomalous sink, which may be explained by the reduced upwelling and associated cessation of the outgassing of CO₂ in the equatorial waters of the Pacific Ocean. On land, the isotopic record implies a concomitant anomalous source, reflecting the effect of the anomalous climate on the terrestrial carbon reservoirs. Interestingly, during the 1992 ENSO event this standard pattern of opposing anomalous sources and sinks is no longer observed. Apparently the climate anomalies, and in particular the cooling in mid-latitudes induced by the eruption of Mount Pinatubo, suppressed the anomalous land source and may thus be partly responsible for the reduced rates of increase in atmospheric CO₂ observed during 1992 and 1993.

How reliable is this information? Over the past 20 years, networks of stations for the measurement of atmospheric CO₂ have been established, and they confirm the observations from Mauna Loa Observatory and the South Pole. Using data from stations suitably placed over the globe, it is even possible to provide coarse information on the geographical latitude of the anomalous sources and sinks, at least for the past decade⁷.

With respect to the isotopic composition of atmospheric CO₂, however, Francey *et al.*⁸ have published a conflicting record from Cape Grim Observatory in the Southern Hemisphere which implies that the global carbon cycle is much more quiescent, in particular on the ENSO timescale. The discrepancy between the two sets of results is discussed by Keeling *et al.* at the end of their paper, and whether it reflects differences in analytical technique, measurement problems or peculiarities of the observing stations is still an open question. That will have to be sorted out — after all, at least for the shorter timescales, these interannual variations in atmospheric CO₂ concentration are the only way in which we can quantify climate feedbacks on the global carbon cycle. □

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Footnote on limb evolution

Craig E. Nelson and Cliff Tabin

MACROEVOLUTION, the generation of morphological novelties in the evolution of new species, stems from alterations in developmental programmes. The relatively recent identification of highly conserved developmental regulatory genes raises the exciting possibility that comparative molecular embryology can be used to elucidate the molecular basis of macroevolution. The past year has seen papers addressing the relationship between the activity of regulatory genes and morphological variation in arthropod appendages and segmentation, as well as in the vertebrate axial skeleton^{1–4}. On page 678 of this issue⁵, Sordino and co-workers use this approach to gain insight into long-standing questions of the evolution of the tetrapod limb — in particular, the fin-to-limb transition.

The authors have analysed the expression patterns of some of the *AbdB*-related *Hox* genes in the developing fin bud of the teleost fish *Danio rerio* (the zebrafish);

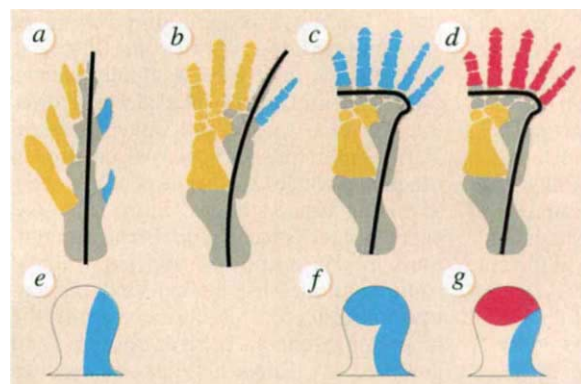
these genes are also known to be important in the growth and patterning of the tetrapod limb. The mesoderm of the zebrafish fin bud displays posteriorly nested expression of the *Hoxd* genes (*e* in the figure) *Hoxd-10*, *Hoxd-11*, *Hoxd-12* and *Hoxd-13*, and uniform expression of *Hoxa-11*. In this respect the fin bud looks very much like the early developing tetrapod limb bud. What is most significant, however, is that Sordino *et al.* find that the fin bud does *not* display the *Hox* gene expression patterns of the late limb bud.

The developing tetrapod limb undergoes at least two temporally and spatially distinct phases of *Hox* gene expression (*f* in the figure). The last of these phases occurs in a spatial domain which correlates with the anlage of the hand or foot (formally known as the autopod but, for simplicity, referred to here as the foot). The zebrafish fin bud, in contrast, never shows this last phase of expression characteristic of foot development. Rather, it

only displays the phase of expression associated with more proximal elements of the tetrapod limb (the tibia and fibula, or zeugopod). Thus, at the level of *Hox* gene expression, the foot plate of the embryonic tetrapod limb has no homologous structure in the developing zebrafish fin bud. The lack of development of fin structures homologous to the foot provides molecular evidence supporting a long-standing school of thought that holds that the tetrapod foot arose as an evolutionary novelty.

This idea was first proposed by Holmgren more than sixty years ago⁶. As a result of extensive comparative morphological analysis of developing limb buds, Holmgren argued that digits are unique and defining to the tetrapods. This view differed from the conventional thinking of the time, which homologized radials of fins to the digits (*a* and *b* in the figure), and had both the radials and the digits branching anteriorly and posteriorly from the main axis of cartilaginous condensation called the metapterygial axis.

In a landmark paper some 50 years later, Shubin



Comparison of postulated metapterygial axis and *AbdB*-like *Hoxd* gene expression in primitive fish fins and tetrapod limbs; anterior is left and proximal is down. *a*, Representative skeleton of primitive fish fin showing radials branching anteriorly (yellow) and posteriorly (blue) from the metapterygial axis. *b*, Representative primitive tetrapod limb with the metapterygial axis as defined prior to Shubin and Alberch⁷. Note that the digits branch both anteriorly (yellow) and posteriorly (blue) from axis. *c*, Tetrapod limb with axis as redefined by Shubin and Alberch⁷. Note the progression of the metapterygial axis through the digital arch. The tibia is considered to branch anteriorly (yellow) from the axis. All digits, however, branch posteriorly (blue). *d*, Alternative model for the development of the tetrapod limb with axis as defined by Shubin and Alberch⁷. Here the digits (red) are considered truly neomorphic and are not homologous to the posterior radials of the primitive fish fin. *e*, Hypothetical *AbdB*-like *Hoxd* gene expression pattern (blue) in the fin bud of the primitive fish based upon zebrafish *Hoxd* gene expression as reported in Sordino *et al.*⁵. The *Hoxd* expression border follows the metapterygial axis in *a*. *f*, Two phases of expression of a representative *AbdB*-like *Hoxd* gene (blue) in the tetrapod limb bud. The border of expression follows the metapterygial axis as defined by Shubin and Alberch⁷ (*c*). *g*, Two independently regulated phases of a representative *AbdB*-like *Hoxd* gene. One phase (blue) is homologous to expression postulated in the primitive fish fin bud. The other phase (red) is neomorphic.

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and Alberch⁷ extended and elaborated Holmgren's hypothesis in a comparative morphogenetic analysis of limb development. Shubin and Alberch redefined the metapterygial axis as bending anteriorly through the distal carpals (the digital arch) and not continuing distally through any given digit (*c* and *b* in the figure). As a consequence of this redefinition of the axis of the limb, the digits are considered to be homologous to the posterior radials of an ancestral fin (*a* and *c* in the figure). In this formulation, the branching behaviour of the cartilaginous condensations that form the limb skeleton changes dramatically between the formation of the forearm and the hand. This particular change in branching behaviour has not been observed during the development of any non-tetrapod appendage, suggesting that this condensation pattern is the result of the invention of a new developmental programme, and again implying that the foot arose as an evolutionary novelty. In 1991, Coates⁸ noticed that the distinct phases of *Hoxd* gene expression correlate with the organization of the limb as proposed by Shubin and Alberch (*c* and *f* in the figure).

In their comparative study of *Hox* gene expression in zebrafish fin buds, Sordino *et al.*⁵ now provide dramatic molecular evidence in support of Holmgren, and Shubin and Alberch. The distinct lack of *Hox* expression characteristic of the tetrapod foot in the developing fin bud suggests that the teleost possesses no structure homologous to the foot, and that the foot was added to the limb after the divergence of the teleost and tetrapod lineages. However, the teleost fish are a highly derived group, having diverged significantly from the primitive fish, and their fins are not necessarily representative of the morphology of the fins of a common ancestor to the teleosts and tetrapods. So a stronger test of the novelty of the tetrapod foot would be to examine *Hox* gene expression during fin development in primitive fish as well as fin buds of our closer relatives the lungfish and (if embryos could be obtained) the coelacanth. Examination of these patterns of expression might also contribute to the resolution of the contested phylogenetic

relationships of these fish to the tetrapods.

As well as providing a molecular character for phylogenetic analysis, the *Hox* genes, as regulators of developmental patterning, provide a potential mechanistic explanation for the evolution of the foot. Mutational analysis of the *Hox* genes suggests that one of their major roles may be to regulate rates of cellular proliferation⁹. Fate mapping indicates that the autopod (the foot) develops from a small posterior distal domain of the early limb bud^{10,11}. So the establishment of a new posterior/distal phase of *Hox* gene expression may have been pivotal in the evolutionary innovation of the autopod.

A key evolutionary and developmental question is raised by these data. Is the terminal phase of *Hox* gene expression an

anterior distal expansion of the previously established posterior expression domain (*f* in the figure), representing a true bending of a pre-existing axis (*c*)? Or does the final *Hox* phase represent *de novo* activation of the same *Hox* genes in a new domain (*g*), in which case the digits would be completely novel tetrapod inventions not homologous to posterior fin radials (*d*)? Further work on the function of the *Hox* genes and their regulation during the different phases of limb development will address this question and provide a deeper understanding of the evolution and development of the tetrapod limb. □

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MUSCLE CONTRACTION

Crossbridge tilting confirmed

Andrew F. Huxley

INNUMERABLE theories have been proposed for the origin of the force that causes relative sliding of the thick filaments relative to the thin when a muscle contracts. There is evidence, accepted by most workers in the field, that the heads of myosin molecules, projecting from the thick filaments, attach to an adjacent thin filament and act more or less independently as force generators distributed throughout the zones in the striation pattern where the two types of filament overlap. A report by M. Irving and others on page 688 of this issue¹ takes an important step in providing direct evidence for a tilting movement of the myosin head during the working stroke, which has for many years been the most popular hypothesis for the actual production of force by an attached crossbridge.

The first suggestion that the crossbridges may act in this way² came from electron micrographs of longitudinal sections of 'asynchronous' flight muscles of insects, a type of muscle with exceptionally regular structure: in rigor, a state probably similar to the final stage of a working stroke, the crossbridges were seen to be at an angle of about 45° to the long axis of the fibre, whereas at rest they were more nearly at 90°. This idea was developed in an influential article by H. E. Huxley³ which suggested specifically that force was developed by a tendency for the myosin head to rotate about its attachment to the thin filament, thereby acting as a crank and transferring force to the shaft of the thick filament through the part of the myosin molecule known as 'subfragment 2' (or S2) acting as a connecting rod.

When a small sudden change of length is applied to a single muscle fibre during

contraction, the accompanying change of tension is largely reversed during the following 1–2 milliseconds (ref. 4), an event generally recognized as representing (in some sense) the 'working stroke'. Structural changes underlying this event have been studied in recent years by X-ray diffraction^{5,6} and by birefringence measurement⁷ and have been readily interpreted in terms of tilting of the myosin heads, but these methods are not highly specific for rotation as opposed to other possible changes in the contractile apparatus. On the other hand, the method used by Irving *et al.*¹, the polarization of fluorescence, is insensitive to almost any change except in the orientation of the fluorophore.

This approach was first used more than twenty-five years ago⁸ but Irving and colleagues have developed it in many ways so as to make it more specific. Thus the fluorophore, a rhodamine dye modified to make it attach to sulphhydryl groups, was applied not to the whole muscle fibre or even to whole myosin molecules, which contain several sulphhydryl groups, but to one of the light chains of a myosin (from chicken gizzard) that contains only one sulphhydryl group. This was exchanged for the original light chain of the muscle fibre (from rabbit) under investigation. This component of the myosin molecule is located in the region thought to be most likely to act as the crank in propelling one filament relative to the other⁹. Changes in orientation were found both simultaneously with the imposed length change and during the 'working stroke', which follows immediately. In a release, the change was in the direction that brings the axes of the fluorophores more nearly

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perpendicular to the fibre axis.

Unfortunately, there are still many uncertainties in the interpretation of such measurements. In the first place, the angle between the absorption and emission dipoles is not known, though it is believed to be small in the rhodamine dyes. A more serious limitation is that the orientation of the dipole(s) relative to the long axis of the myosin head is unknown. Also, the polarization signals show that there is large variation in the angles made between the dipoles and the fibre axis, and this may have many causes: a proportion of the heads being randomly oriented (perhaps not attached to actin); there being two or more populations of heads with different orientations; and the fluorophore being capable of limited rotation relative to the myosin to which it is attached.

A different kind of difficulty is that the movements appear to be rather small, totalling not more than about 3° of tilt with a release of 3.5 nanometres per half-sarcomere. Of this 3.5 nm, some will have been lost in the compliance of the filaments because the original tension is not completely recovered during the working stroke, so the relative movement of thick relative to thin filaments in each overlap zone was probably about 3.0 nm. However, the effective length of the crank is probably between 10 and 20 nm, so the

movement corresponding to 3° is about 0.5–1 nm. Of this, about two thirds would be simultaneous with the length change and one third accompanies the subsequent recovery of tension. As regards the elastic part of the response, which is simultaneous with the length change, this is not a difficulty: it is often assumed that much of the compliance lies in other parts of the myosin molecule (for example, the S2 neck region), and there is now much evidence^{10–12} that about half of the instantaneous compliance resides in the filaments, not in the crossbridges. As regards the working stroke itself, about two thirds of the initial tension drop was recovered during this phase, so if there had been no active tilt of the crossbridges, the elastic change would have dropped to one third, while in fact there was further movement in the same direction; the tilt attributable to the working stroke is the difference, namely up to 2° or so.

It has always seemed likely⁴ that there is more than one component in the 'working stroke', so perhaps tilt of the myosin head is only one of these. Alternatively, the dipole axis may not be perpendicular to the axis about which tilt occurs, so that only a part of the tilt is registered by the changes in polarization, or there may be flexure between the tilt axis and the attachment of the fluorophore.

The interesting observation that the direction of the polarization response is reversed when the fibre is put into rigor implies that the mean tilt of the myosin heads is on the opposite side of the perpendicular to the fibre axis, in agreement with the electron microscope observation that first suggested tilting of the heads².

In any case, this is a most encouraging start of a new approach to the central problem of muscle contraction, and all muscle physiologists will look forward to its development. □

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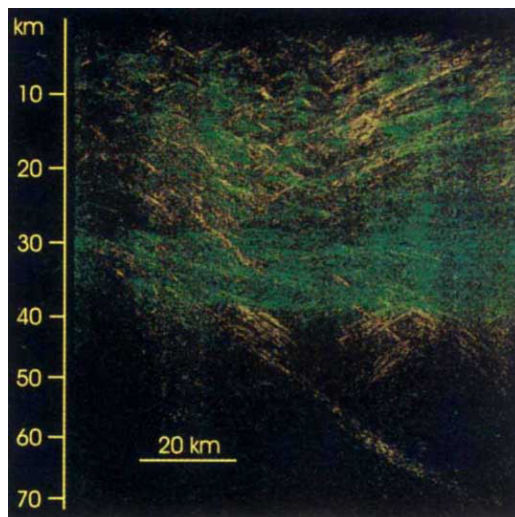
EARTH HISTORY

Reflections on plate tectonics

THE Earth today is a well regimented planet, with its surface divided into a small number of lithospheric 'plates' that slide as coherent masses over the mantle beneath. New oceanic lithosphere is formed at spreading ridges, cools and thickens, and disappears back into the mantle at deep-sea trenches; the result is a rather efficient escape mechanism for the Earth's internal heat. But this isn't the only way for a planet to organize its affairs. In particular, it has been suggested that the hot early Earth may have favoured a more chaotic tectonic style — for example, with small blocks of lithosphere foundering incoherently into the mantle, instead of the subduction process that we see at trenches today.

Uniformitarians everywhere will accordingly take heart from the seismic cross-section shown here, obtained by Calvert *et al.* from a 2.7-billion-year-old terrain in Canada's Superior Province and discussed on pages 670–674 of this issue. The steeply dipping seismic reflections (yellow) that extend to 65 km depth mark a shear zone along which oceanic crust was thrust down into the mantle (the

unreflective region below about 40 km). This region of the Superior Province had already been identified as a collision zone from its surface geology; the seis-



mic profile now suggests that this collision followed the disappearance of an ocean basin, by a process indistinguishable from modern-day subduction.

The seismic data reveal another modern feature of this Archaean terrain: the presence of a strongly reflective

lower crust (sub-horizontal green lines between 30 and 40 km depth). The absence of this feature in previous seismic images of Archaean terrains has led to suggestions that Archaean continental crust might have formed in a different manner from its more recent counterparts. Now it seems that in at least some of these other terrains an originally reflective signature in the lower crust may have been lost by subsequent modification — perhaps due to a heating episode.

The new results extend the evidence for modern-style plate tectonics back into the Archaean aeon — the earliest subdivision of Earth history — beating by 800 million years the previous record of 1.9 billion years, held by a collision zone in the Proterozoic Baltic shield. How much further back might it go? Terrains older than 3.5 billion years are now known from several continents, and the oldest intact rocks date back to almost 4.0 billion years, so there is scope for yet another significant leap backwards.

Laura Garwin

Laura Garwin is Physical Sciences Editor of *Nature*.

Seeing and believing

Jules P. Halpern

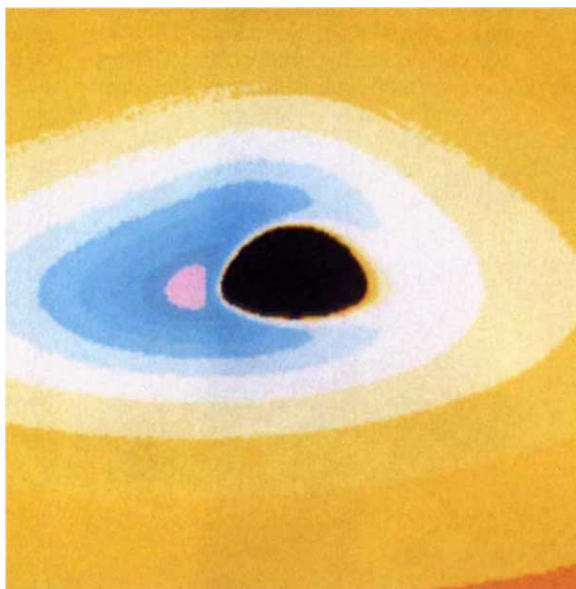
THE most productive year yet for the pursuers of black holes has been crowned by a long-awaited discovery that represents the closest we have come to actually seeing one. On page 659 of this issue¹, Tanaka *et al.* report that a severely distorted emission line, seen in the X-ray spectrum of a Seyfert galaxy, is of exactly the right shape to be coming from the innermost regions of an accretion disk around a supermassive black hole. The bright, 'active' nucleus of a Seyfert galaxy is thought to be powered by the gravitational potential energy of material spiralling slowly inwards through an accretion disk, into a black hole of more than a million solar masses. The shape of an emission line from such a structure — in this case the $K\alpha$ fluorescent line of iron at an energy of 6.4 keV (wavelength 1.94 Å) — provides the most direct evidence for the enormous velocity approaching the speed of light, and other manifestations of the strong gravitational field that controls the appearance of the disk in the immediate neighbourhood of the black hole's 'event horizon'.

If one could view an accreting black hole in visible light, it would look something like the accompanying figure². The rotating accretion disk, rather than appearing as the symmetric annulus around a dark centre that it really is, looks distorted. The gravity of the black hole bends the rays of light passing around it. Also, the side of the disk moving towards the observer looks brighter than the side moving away, a result of the so-called headlight effect for rapidly moving sources of light. With colour vision, we would see the Doppler shift, with the approaching side of the disk blue, and the receding side red.

We will probably never see this picture directly because, at the great distances of the galaxies, the accretion disk, which is comparable in size to the Solar System, is too small to be resolved. Nevertheless, the iron $K\alpha$ line seems to be confined to the inner accretion disk where conditions are uniquely suited to its production. Spectroscopic measurements of its profile (intensity versus wavelength) can be even more revealing of the black hole's extreme properties than a visible image, because the spectrum contains information about the velocity of the material in the disk as well as the time dilation (gravitational redshift) that causes the atoms' clocks to run slower in the gravitational field of the

black hole. Theoretical predictions of the line profile corresponding to this picture have been discussed optimistically for the past six years or so³. Advances in X-ray spectroscopy put into practice by the Japanese-American satellite ASCA, and dedicated long exposure times, made possible the corresponding observational discovery reported by Tanaka *et al.*¹.

Both the inclination angle of the accretion disk to the line of sight, and its radius relative to the radius of the black hole's



Simulated picture of an accretion disk around a black hole, from ref. 2, viewed at an angle of 20° above the disk plane. The sense of rotation is left side approaching and right side receding. The observed temperature, shown in false colour, ranges from $<3 \times 10^6$ K (orange) to $>1.2 \times 10^7$ K (violet).

event horizon, are determined by fitting the spectrum to a simple model. One might try to dream up alternative explanations for the peculiar shape of the line that do not involve a black hole, but I agree with the authors that the detailed properties of this particular spectrum are difficult to rationalize in any other way. Just about the only quantity that has not been measured by this observation is the mass of the black hole, for the simple reason that material falling in has the same velocity no matter what the black hole's mass is. It is probably 10^7 solar masses, and future, more sensitive instruments should be able to measure it because time variability of the line profile can reveal the absolute size of the disk, which is proportional to the mass of the black hole and possibly only a few light-minutes in radius. With the acquisition of higher quality data, it may even be possible to learn whether or not the black hole itself is rotating, not to mention details of accretion

disk structure never seen before.

The search for a proof of the existence of supermassive black holes has long followed two complementary paths. The first method is the one described above, which now appears to have reached fruition. The second method is an older one. It involves observation and modelling of galactic rotation and random velocities using the starlight spectrum in the centres of nearby, non-active galaxies such as Andromeda and NGC3115, in order to deduce the presence of dark, gravitating mass that cannot be accounted for by the amount of starlight present. The existence of black holes of 10^7 – 10^9 solar masses has been inferred in this way⁴. More recently, this stellar dynamics method has been applied in a simplified form to the observations of maser emission with radio interferometers⁵ and visible line emission with the Hubble Space Telescope⁶, using the apparently circular orbits of these sources to demonstrate the need for supermassive black holes in regions as small as one light year across at the centres of active galaxies.

The two methods are orthogonal in the following sense. The X-ray emission line offers the opportunity to 'see' a black hole through the unique relativistic effects of strong gravity; but because the emission arises from gas, which is susceptible to forces other than gravity, it cannot prove rigorously that a black hole is responsible. The stellar dynamics method, on the other hand, is immune from the effects of non-gravitational forces. However, it demonstrates the existence of a supermassive black hole without seeing it, because it operates in a region far

larger than any in which general relativity could produce observable effects that are distinct from ordinary newtonian gravity. Perversely, we can either see a black hole, or prove it exists, but not both! Which method is more satisfying is ultimately a matter of taste. Probably because of this fundamental complementarity, practitioners of either approach hardly ever acknowledge the others. Perhaps they will — starting now. □

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Replenishing the stores

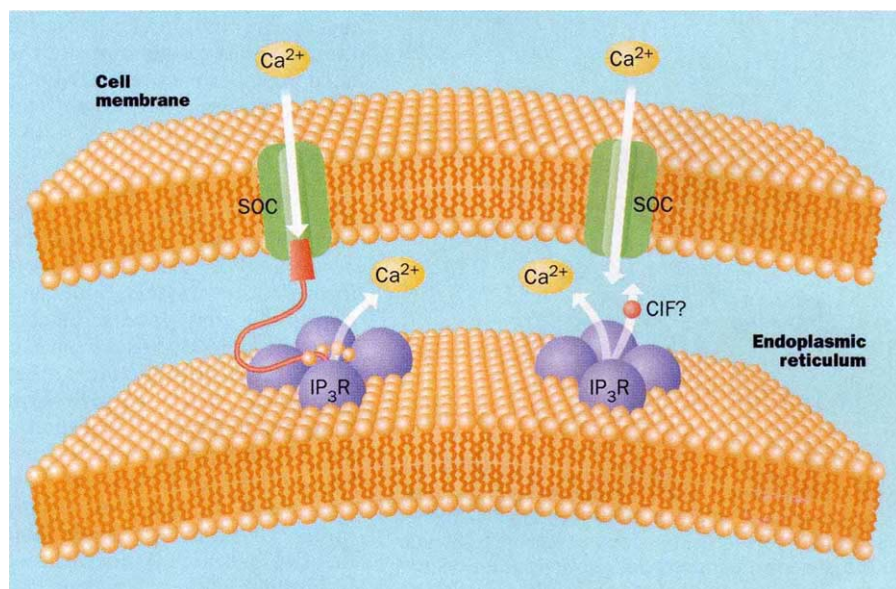
David E. Clapham

HOPES are waning for the isolation and identification of the calcium-influx factor (CIF), a possible second messenger that regulates the entry of calcium ions into the cell in order to keep the calcium stores topped up. At a meeting held last month on receptor-regulated calcium influx*, these doubts that CIF is in hand were overtaken by new hopes for the identification of a possible calcium-store-operated channel following the announcement of the cloning of the human homologue of the *Drosophila* gene *trp*.

Dozens of G-protein-linked or tyrosine-kinase-linked receptors initiate a cascade

One of the most interesting unsolved mysteries in biology is why entry of calcium ions at the plasma membrane invariably follows calcium release from the endoplasmic reticulum^{2,3}. Besides sustaining calcium and repleting stores, this mechanism maximizes calcium near the membrane where specialized calcium-dependent proteins are found.

The question that has driven research in this field is how the emptied endoplasmic reticulum stores manage to open the plasma membrane Ca^{2+} floodgates. There are two competing hypotheses: the first presupposes a direct link between the recep-



Two possible ways in which calcium ions could be induced to enter a cell in response to a depletion of stored calcium in the endoplasmic reticulum. In the scheme depicted on the left, there is a direct link between the receptor for inositol 1,4,5-trisphosphate (IP₃R) and a putative store-operated calcium channel (SOC) at the plasma membrane. In the alternative scheme shown on the right, this channel is controlled by a diffusible second messenger which is released from the endoplasmic reticulum and may be the elusive calcium-influx factor, CIF.

that generates intracellular inositol 1,4,5-trisphosphate (InsP₃) to release calcium ions from the endoplasmic reticulum inside the cell¹. Calcium acts as a ubiquitous, powerful trigger of conformational change in many proteins to initiate functions as diverse as muscle contraction, secretion and neurotransmission. InsP₃ receptors are gatekeepers of the calcium store in the endoplasmic reticulum and InsP₃-mediated intracellular calcium release is common in non-excitable cells such as lymphocytes. (In excitable cells like neurons, voltage-activated calcium-selective ion channels located in the plasma membrane provide an additional, faster route for raising intracellular calcium.)

tor for InsP₃ and a putative surface Ca^{2+} channel (see figure, left), analogous to the skeletal muscle ryanodine receptor/dihydropyridine receptor dyad; the second is that a diffusible second messenger released from the endoplasmic reticulum gates a Ca^{2+} channel at the plasma membrane (right in the figure). Two key developments have titillated investigators in the area — the characterization of a depletion-activated calcium-selective current (I_{CRAC}), identified by Lewis and Cahalan^{4,5} and Hoth and Penner⁶, and the partial characterization by Randriamampita and Tsien⁷ of a calcium-influx factor.

At this meeting it became clear that I_{CRAC} is expressed in many different cell types. Its principal attributes are its activation by any mechanism that depletes in-

tracellular calcium stores (for example by ionomycin, calcium chelators, InsP₃ or thapsigargin), a selectivity higher than 1,000:1 for calcium over potassium or sodium, a low unitary conductance (below 100 femtosiemens), and voltage independence. It is not activated by cyclic AMP or cyclic GMP, or by GTP, InsP₄ or a variety of small G proteins (R. Penner, Max Planck Inst. for Biophysical Chemistry, Göttingen), and is potentiated by extracellular calcium but inactivated by intracellular calcium (A. Zweifach and R. Lewis, Stanford Univ., California). I_{CRAC} is not the only type of channel bringing Ca^{2+} into cells that is triggered by store depletion; such channels are known generally as store-operated channels (reviewed in refs 1 and 8). An interesting swelling-activated Ca^{2+} -carrying current was described in immature thymocytes, accompanied by a dramatic video showing the role of calcium in T/B-cell interaction (M. Cahalan, Univ. of California at Irvine).

One of the most promising candidates for an I_{CRAC} -like protein is the mammalian homologue of the *Drosophila* protein Trp. Trp appears to be a key element in the InsP₃-dependent phototransduction process in invertebrates. Mutants lacking Trp show only a transient receptor potential (hence *trp*) in response to light which is due to a defect in store-depletion-gated calcium entry. These tantalizing features have led investigators to express *trp* in cells (D. Kunze and W. Schilling, Baylor, Texas) in order to investigate its electrophysiological characteristics. Expressed Trp has properties that are consistent with store-operated channels, although it is not necessarily I_{CRAC} itself. Such findings have spurred several laboratories to clone mammalian homologues of *trp*, the first of which was reported at the meeting (C. Montell, Johns Hopkins Univ., Baltimore). Human Trp is one of a family consisting of at least three other members and is found at highest levels in brain, heart, testis and ovary; it is some 450 amino acids shorter than *Drosophila* Trp at the carboxy terminus. The putative six-transmembrane-spanning-domain protein has regions homologous to sequences found in ankyrin and dystrophin and resembles an ion-channel structure. So far, human *trp* has not been functionally expressed.

Probably the widest interest at this meeting was aroused by the moribund state of calcium-influx factor as the potential second messenger between depleted stores and I_{CRAC} or related store-operated channels. CIF was originally proposed to be a small (relative molecular mass 0.5K), anionic, phosphorylated compound which was prepared from cell extracts that caused the influx of Ca^{2+} when applied to various cell lines⁷. Little progress has been made by these authors (R. Tsien, Univ. of

*International Conference on Receptor-Regulated Calcium Influx, Asilomar, California, USA, 14–18 May 1995.

California at San Diego; C. Randriamampita, Ecole Normale Supérieure, Paris) in identifying CIF, and attempts to activate bona fide I_{CRAC} with the extract have been unsuccessful. It was shown (G. Bird and J. Putney, National Inst. of Environmental Health Sciences, North Carolina) that such extracts are complex and contain a variety of agents that may release Ca^{2+} by more conventional means. Another, related extract (M. Hanley, Univ. of California at Davis) tested by B. Premack (Stanford Univ.) was found to activate an unrelated non-selective cation conductance. The long-awaited influx factor is unlikely to materialize just yet.

The alternative, direct coupling between the $InsP_3$ receptor and an ion channel received oblique support (C. Petersen and M. Berridge, Cambridge Univ.) from a clever approach that separated regions of a store-depleted *Xenopus* oocyte and found no evidence for a diffusible factor. A partial *trp*-like sequence was found in oocytes and *Drosophila trp* ex-

pression in oocytes enhanced oocyte store-operated channels. Evidence against direct coupling comes from experiments with T cells deficient in $InsP_3$ receptors. Although there was no receptor-mediated mobilization of calcium, the cells still allowed store-depleted Ca^{2+} entry⁹. Proof of a direct coupling between the $InsP_3$ receptor and the Ca^{2+} entry channel is not imminent either. □

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NEUROBIOLOGY

New visions of the cortex

Charles Jennings

EVER since Hubel and Wiesel first began to explore the primary visual cortex with a recording electrode more than 30 years ago, our view of how the brain works has been dominated by studies on the visual system. As was clear from a recent meeting*, the trend continues to this day. Increasingly, however, lessons from the visual system are also illuminating our understanding of other cortical regions.

From the retina, visual information is conveyed via the thalamus to the primary visual cortex, and from there it passes to a myriad of higher cortical areas. Neurons at early stages in the pathway respond to simple features such as spots of light, but at each subsequent level the information is reanalysed, so that cells at later stages can detect higher-level features such as line orientation, movement, colour and even specific objects.

Mapping these pathways is a major task, but nearly forty distinct visual cortical areas have now been identified. Imaging techniques such as positron emission tomography and functional magnetic resonance imaging allow these areas to be mapped in humans (G. Orban, Katholieke Univ., Leuven; R. Tootell, Massachusetts General Hospital; B. Gulyas, Karolinska Inst., Stockholm), and in many cases the human data can be related to anatomical and physiological data from equivalent areas in monkeys — although,

as pointed out by A. Cowey (Univ. of Oxford), the functional correspondences are not always clear-cut.

After the primary visual cortex, visual information is, broadly speaking, channelled into two parallel pathways, a dorsal pathway that is concerned with movement and location of stimuli, and a ventral pathway concerned with stimulus qualities such as shape, colour and object recognition. These have been described as the 'where?' and 'what?' pathways. Two populations of visual neurons have been identified in the thalamus, the large magnocellular neurons that respond selectively to movement and contrast, and the smaller parvocellular neurons that respond to colour and fine spatial patterns. An attractive notion is that these two populations feed into the 'where' and 'what' pathways, respectively. But as J. Maunsell (Baylor College of Medicine, Texas) showed, this is simplistic; although cortical area V5 in the dorsal pathway is largely driven by magnocellular neurons, area V4 within the ventral pathway shows no such selectivity, and is driven by both populations. How the two pathways interact to produce a unitary perception is not known.

Orban commented that there is a tendency to think of the visual system as an extension of the retina, with the higher cortical areas obediently analysing whatever information the retina sends them. But in fact the cortex plays a much more active role than this, and the same

visual stimulus can activate different areas depending on whether subjects are instructed to view the stimulus passively or to examine it for specific features (Orban). Experiments in monkeys show that the first cortical area in which such effects are clearly manifested is MST (Maunsell); cells in this area respond to dots moving in specific directions, but the strength of the response to a given dot depends on whether the monkey is attending to that dot rather than others. Presumably this effect must depend on connections to MST from other parts of the brain that control attention, but these have not yet been identified.

At some point visual information must be converted into behavioural decisions, and W. Newsome (Stanford Univ. School of Medicine, California) has begun to dissect this process. Monkeys are trained to move their eyes left or right depending on whether they perceive leftward or rightward motion in a pattern of dots. As the task is made more difficult by adding randomly moving dots, the monkeys' performance deteriorates to chance levels; nevertheless, they must still make a choice, and Newsome has recorded from cells in area LIP whose activity predicts, several seconds in advance, which way the decision will go. The activity of these cells builds up gradually as the monkey tries to discern the signal from the noise, and the stronger the signal, the faster the cells reach their maximum firing rate. Thus, activity within LIP does not correspond simply to sensory input or to motor output; rather, it lies somewhere between the two, as would be expected for cells that integrate sensory signals to control behavioural decisions.

Discovering what a given cortical area does is only the beginning; a much greater challenge is to explain how it works in terms of its underlying circuitry. For instance, how does orientation selectivity arise in the primary visual cortex? In principle, this could be explained simply in terms of the pattern of thalamo-cortical connections; if (say) three thalamic neurons whose receptive fields lie in a straight line all converge on the same cortical cell, that cell will respond best to a line that stimulates all three thalamic cells simultaneously. An alternative view, however, is that intra-cortical connections are required to generate orientation selectivity. Attempts to distinguish between these possibilities have led to contradictory results (U. Eysel, Ruhr Univ., Bochum; D. Ferster, Northwestern Univ., Illinois), and given the complexity of the connections involved, explaining even the simplest forms of cortical processing in terms of neuronal circuitry remains a challenge.

One approach to understanding cortical function is to build models of small patches of cortex, based on the known electrical

*Cerebral Cortex Function and Development, INSERM meeting, Lyon, France, 10–13 May 1995.

properties and connectivity patterns of its component neurons. Such models could in principle account for extraction of robust signals from noisy input (R. Douglas, MRC, Oxford), and even suggest a mechanism for working memory in which a pattern of activation is maintained in the absence of the input that triggered it (C. Koch, Caltech).

Is there any such thing as a canonical cortical circuit, and can lessons from the visual system be extrapolated to other cortical areas and functions? Those in search of a general theory of cortical function will be encouraged by the work of M. Sur (MIT), who has found a surprising degree of functional similarity between visual and auditory cortex. If visual inputs are surgically redirected to the auditory cortex of young ferrets, they form a topographical map in which individual cortical cells show orientation selectivity, a property that has no known counterpart in normal auditory cortex. Remarkably, the animals still interpret signals from the operated eye as light rather than sound, even though they are being processed by auditory instead of visual cortex.

Another striking example of functional plasticity is seen in the case of language, often regarded as the most specialized of human cognitive skills. Although certain brain areas are specialized for language production, children who suffer strokes to these areas often recover normal language abilities, indicating that their function can be taken over by other areas (E. Bates, Univ. of California, San Diego). And H. Neville (also UCSD) found that people who have been deaf since early childhood, and who communicate in sign language, show different patterns of cortical activation from those of hearing people during both visual and linguistic tasks.

Although such wholesale rewiring may be peculiar to early development, cortical plasticity can also be demonstrated in adulthood. S. Florence (Vanderbilt Univ., Tennessee) showed that limb amputation leads to extensive changes in subcortical somatosensory connections, but that these are largely corrected by compensatory changes in the cortex. M. Merzenich (Univ. of California, San Francisco) found that even in normal animals, connections can be greatly modified by experience; thus, if a monkey's hand is repetitively stimulated over a period of several weeks, the map of the hand on the somatosensory cortex is altered. The changes are consistent with a hebbian model in which parts of the hand that are stimulated in synchrony form connections to the same cortical site, while segregating away from others that are stimulated out of synchrony.

Again, however, it is in the visual system that plasticity has been studied in greatest detail, and altering the pattern of thalamic inputs to the primary visual cor-

tex produces profound changes in the representation of the visual field within the cortex (M. Stryker, Univ. of California, San Francisco; C. Gilbert, Rockefeller Univ.; R. Freeman, Univ. of California, Berkeley; Y. Chino, Univ. of Houston). Some of these changes are long-term and depend on anatomical reorganization, but others are very rapid; if for example a small patch of cortex is temporarily deprived of visual input (by introducing a dark spot in the visual field), cells in the deprived cortical region begin within seconds to respond to inputs from adjacent parts of the visual field. The underlying cellular mechanism has not been established, however, and it remains unclear whether the observed effects are due to change in the shapes of the cortical receptive fields (Gilbert) or to a more general increase in the excitability of the deprived cortical cells (Freeman).

Clearly, the cerebral cortex is no longer the uncharted territory that it was a few decades ago, but will current techniques and concepts be sufficient to sustain the present rate of exploration? It is clear that some major obstacles lie ahead: for example, W. Singer (Max Planck Inst., Frankfurt) presented evidence that perceptual binding, the process by which multiple elements are combined into a single perceptual entity, may involve synchronous firing of large populations of cells. Such patterns cannot be revealed by metabolic imaging or single cell recordings, and can only be studied using multiple electrodes, a challenging technique that is still confined to a few laboratories. Moreover, some basic issues are still obscure; for instance, as H. Barlow (Univ. of Cambridge) pointed out, timing is an essential component of both perception and behaviour, yet we still have little idea of how time is represented in the brain. Finally, conspicuous by its absence at this meeting was any discussion of the frontal lobes or their role in 'higher' cognitive functions. Human behaviour is of course determined not only by sensory inputs, but also by emotions, knowledge and reason, and understanding how all these factors interact to control behavioural decisions remains a very distant goal.

The brain is of course a product of development and ultimately of evolution, and to understand how intelligence evolved, we will need to understand not only how the brain works, but also how its development is controlled by the genome. As was clear from many other talks at the meeting, our knowledge of brain development is advancing rapidly, but the underlying genetic mechanisms are still largely mysterious. It will be interesting to see if the great synthesis looks any closer by the millennium. □

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DAEDALUS

Carbon in chains

A FEW years ago Daedalus proposed to make the long-sought linear polymer of carbon, carbyne, by rapid quenching of hot carbon vapour (*Nature* 360, 22; 1992). A recent synthesis using just this method has been dignified by editorial mention in these columns (J. Maddox, *Nature* 375, 11; 1995). Other syntheses have been reported, and the topic is developing rapidly. Carbyne is unstable, and probably explosive in bulk. Daedalus hopes to tame it by alloying it with metal.

He points out how easily graphite forms compounds with metals. Metal atoms readily get into the electron-rich spaces between its sheets of atoms, where they form collective metal-carbon bonds. Conversely, graphite is quite at home in many metals. Cast iron owes its easy machinability and its high internal damping to its graphite content. Daedalus reckons that carbyne could be stabilized the same way. Its chains of multiple-bonded carbon atoms should happily share some of their energetic π -electron density with a surrounding metal.

DREADCO's chemists are now working to this brief. One group is quenching carbon vapour in the solutions used to deposit metal mirrors; the idea is that the metal atoms will cluster around the carbyne chains as they form. Another is starting from heavy metal acetylides, which are highly explosive when dry. In the wet, however, there seems a good chance of getting them to rearrange quietly, with their C_2^{2-} ions linking into carbyne chains stabilized by their metal atoms. One way or another, the chemists hope to make a sort of metal-carbyne alloy, with parallel carbyne chains glued together by a matrix of metal.

This, of course, would be the ultimate carbon-fibre reinforced metal. Its uniquely short and stiff carbyne bonds should make it immensely strong. Like wood it will have a grain direction; but more isotropic forms, the equivalents of chipboard and plywood, could easily be fabricated. Metal-carbyne wire and laminate will make amazingly light and rigid prestressed structures, suspension bridges of unprecedented span and wonderfully light aircraft. Sadly, the alloys will retain much of the high energy of the carbyne: once ignited, they will probably burn fiercely. So Daedalus is thinking of other uses.

One will almost certainly be electronic. A set of carbyne chains in parallel with metal, and bonded to it by collective π -bonds, should have intriguing anisotropic, nonlinear or semiconducting properties. It might even be the long-awaited room-temperature superconductor! David Jones

HIV clearance in an infant?

SIR — Infants born to HIV-infected mothers acquire maternal antibody transplacentally, rendering the interpretation of serological assays difficult. Secure diagnosis of perinatal infection should include investigation both of a maternal sample and of two or more infant samples, using a combination of assays for the virus (culture, genome and antigen detection) and for the evolving serological response (or its absence) in the infant. For absolute security, results must be concordant at any point in the sampling schedule.

two occasions, 19 and 51 days after birth, and once from a plasma sample taken at 51 days. This diagnosis was not confirmed by any other means, and the only positive PCR (polymerase chain reaction) result at 33 days was unconfirmed by virus culture or detection of core antigen. These results do not constitute a concordant dataset.

Because contaminated cultures and false-positive PCR results are an occupational hazard, we consider a baby to be infected with HIV only when PBMCs and/or plasma taken from the infant on two separate occasions are both culture- and PCR-positive, or to be positive by one of these tests in the face of an evolving antibody response. These stringent criteria remove the element of diagnostic controversy that inevitably surrounds cases of 'viral clearance'. Although ten sequential samples were taken postnatally from the infant described in the paper by Bryson *et al.*, at no time is any one sample shown to be positive by both PCR and culture simultaneously. Hence, it is not unreasonable, in our opinion, to call the baby's virological status into question.

To eliminate the possibility that their results could be explained by inadvertent laboratory contamination of viral isolates, Bryson *et al.* sequenced independent clones of the V4–C4 part of the viral envelope from the HIV-cultured samples. Sequence similarity between the mother and baby isolates is presented by the authors as evidence of an evolutionary relationship between them. Although the two isolates from the baby are indeed closely related, there is nothing in these

sequences to link them with the mother. Our phylogenetic analysis of the published sequences clearly shows that the baby isolates are no more closely related to those of the mother than to many other laboratory strains of HIV (see figure). This, of course, may be the result of selection of a minor variant following culture of the baby's blood sample before sequencing; an extremely rapid turnover in the viral quasispecies of the mother in the year after delivery and before her viral samples were obtained; or a function of

the fact that the short V4–C4 region is not ideal for demonstrating genetic relatedness between mother and baby. We urge Bryson *et al.* to analyse a sequence for which the evolutionary dynamics are more amenable to the demonstration of HIV transmission, such as V3 (ref. 5) or the p17 region of the *gag* gene⁶.

In view of the above considerations, we believe it is premature to claim clearance of the virus by this or any other infant.

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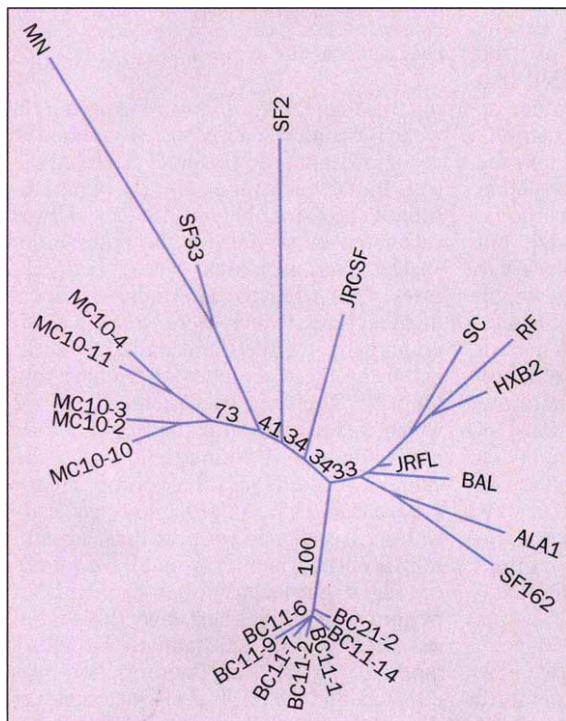
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BRYSON AND CHEN REPLY — We disagree with the interpretations of McClure *et al.* of our recent paper¹ for a number of reasons. The criteria set out by these authors for the diagnosis of paediatric HIV infection are not typical of those used for most paediatric diagnoses. Our diagnosis of HIV infection is based on the presence of cultured virus from the infant on two separate occasions, consistent with the guidelines established by the NIH AIDS Clinical Trials Group Virology Committee and the Centers for Disease



Phylogenetic analysis of mother/child env sequences. DNA sequences corresponding to those published in ref. 1 (BC, infant sequences; MC, maternal sequences; other sequences represent unrelated laboratory strains of HIV) were obtained from GenBank and aligned using CLUSTAL. Modifications to improve the alignment were made by hand. This alignment was used as input for PHYLIP programs. Genetic distance was estimated using the six-parameter model of J. Felsenstein (PHYLIP program DNADIST, version 3.5c; distributed by the author, Department of Genetics, University of Washington, Seattle), and these distances were used to construct a neighbour-joining tree. The robustness of the tree was assessed using the bootstrap resampling method with 100 replications.

Following the media attention surrounding the reported clearance of HIV infection in a perinatally infected infant by Bryson *et al.*¹, it is worth noting that on the data presented, the baby in question would not have met the criteria of infection adopted at some hospitals in the UK, although we accept that they do meet the standards set down by others^{2–4}.

The diagnosis by Bryson *et al.* hinges on virus being cultured from peripheral blood mononuclear cells (PBMCs) on

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2. Caldwell, M. C., Oxtoby, M. J., Simmonds, R. S., Lindegren, M. L. & Rogers, M. F. *Morbid. Mortal. Wkly Rep.* **43**, No. RR-12 (1994).
3. Paediatric European Network for Treatment in AIDS — Trial entry criteria available from the UK Medical Research Council.
4. Rep. 2nd Meet. WHO Informal Working Group on Prevention of Mother to Child Transmission of HIV: Standardization of Techniques and Criteria for the Diagnosis of HIV Infection in Neonates, Rockville, USA (WHO, Geneva, 1995).
5. Ahmad, N., Baroudy, B. M., Baker, R. C. & Chappey, C. J. *Viral.* **69**, 1001–1012 (1995).
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Control^{2,7,8}. We would certainly have liked to have performed PCR on these occasions, but were limited by the small quantity of specimens available in retrospect, particularly given the extremely low viral load (10^6 cells required for each positive culture and three copies of viral DNA per 10^5 cells by PCR at a separate time point).

As this is an unusual case, we undertook more stringent tests to demonstrate infection. We obtained two viral isolates cultured from the infant's blood at time points approximately 1 month apart, allowing us to compare these viruses and thus determine whether inadvertent contamination might have occurred on two separate occasions. Nucleotide sequence analysis of the two viral isolates demonstrated that these viruses are extremely closely related and distinct from other strains of HIV-1, including those commonly used in the laboratory, making contamination in the laboratory highly unlikely.

Given the limited sequence analysis, we did not attempt to establish an evolutionary relationship between the mother's virus and that of the infant (further correspondence arising from our paper will appear soon in *The New England Journal of Medicine*). Although the origin of the infant's virus as maternally derived has not yet been formally proven (studies are currently underway), the most likely route of infection would have been by vertical transmission.

In any event, the origin of the virus has no bearing on our central conclusion, that the infant was infected with HIV — as evidenced by the isolation of highly related virus on two separate occasions — and then became virus-free.

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Errata

In the Scientific Correspondence "Another obese gene function" by M. T. Nakamura (*Nature* **374**, 124; 1995), the second paragraph incorrectly referred three times to normal mice as *ob/-*. This should have read *Ob/-* to denote the fact that the mouse carried at least one dominant normal gene (*Ob*). □

In the Scientific Correspondence "Community response to IRONEX" by K. Banse (*Nature* **375**, 112; 1995), the second sentence of the table legend should have read: "Column 3. Initial, photosynthesis from outside the patch (equivalent to "out" in Fig. 3 of ref. 1), $\mu\text{g C per l per day}$." □

Origin of the Tunguska event

SIR — It is generally accepted that the Tunguska event resulted from the catastrophic disruption of a large meteor high above the ground. Previous studies have yielded diverse interpretations as to the meteor's size, composition, velocity and density before its arrival¹⁻³. Nevertheless, there is consensus that the disruption occurred 6–10 km above the ground⁴, depositing approximately 15 Mton energy⁵ in a narrow altitude band. Turco *et al.*³ concluded that the meteor was of cometary origin with an effective density of 0.003 g cm^{-3} and a diameter of 1,200 m. In contrast, Sekanina concluded that the body was not a comet, but rather an Apollo-type asteroid 90–190 m across². More recent work has suggested that the object was a stony asteroid perhaps 60 m in diameter¹. In the same work, a carbonaceous body was noted as a possible, but unlikely, cause of the event. We argue here that a carbonaceous chondrite was in fact the most probable cause of the event.

The atmospheric trajectory of a meteor is influenced by mass loss due to ablation and fragmentation caused by enormous aerodynamic loads. The equations of motion for such a body have been described elsewhere^{1,2}. An essential aspect of modelling the entry involves accurately calculating the radiation-dominated aerodynamic heating. This can be approximated using the Stefan-Boltzmann relation:

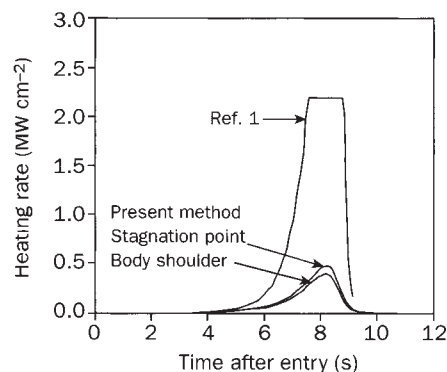
$$q_{\text{Rad}} = 5.67(T_2/1,000)^4 \text{ W cm}^{-2} \quad (1)$$

where q_{Rad} is the adiabatic radiative heat transfer rate, and T_2 is the temperature behind the shock wave (a function of velocity, altitude and shock angle). However, because of radiative emission from the shock layer, the flow is non-adiabatic. This effect can be accounted for following the method of refs 6 and 7:

$$q_R = q_{\text{Rad}} / (1 + 3.33 I^{0.7}) \quad (2)$$

where q_R is the non-adiabatic radiative heat transfer rate and $I = 4q_{\text{Rad}}/(\rho_a V^3)$, where ρ_a is the ambient atmospheric density and V is the meteor velocity. Moreover, ablation products in the shock layer will absorb some fraction of the radiation before it reaches the body. The significance of this effect has been analysed by Gupta⁸ and depends primarily on the ratio of the freestream and ablation product mass flow rates.

We have attempted to determine the type(s) and size(s) of the objects that could have caused the Tunguska event. The equations of motion were numerically integrated for a wide range of bolides and entry conditions, while the body's mass was decreased according to the ablation rate. For asteroids, entry veloci-



Heat transfer rate versus time for a carbonaceous body. Entry velocity, 15 km s^{-1} ; body density, $2,200 \text{ g cm}^{-3}$; entry angle, 45° ; body diameter, 68 m; $C_d = 1.2$.

ties of $12.5\text{--}20 \text{ km s}^{-1}$ were considered, while for comets, entries were examined at the approximate lower limit of 20 km s^{-1} (ref. 9). At each time step, the temperature distribution behind the shock was calculated by solving for the equilibrium species concentrations. These temperatures were used to determine the local heating rates, which were corrected for non-adiabatic effects and radiative blockage using the techniques of Goulard and Gupta^{6,8}. The drag coefficient, C_d , of 1.2 which was assumed for the nominal case corresponds to a blunted ellipsoid. (The value of 1.7 used in ref. 1 is appropriate for a flat-faced, sharp-edged cylindrical body with its axis aligned with the flow. A naturally occurring meteor would have rounded edges, particularly after even a brief period of atmospheric passage; this rounding decreases C_d substantially.) Chyba's model of mechanical deformation was adopted, and the physical characteristics of the bolide materials were based on ref. 1.

The figure shows a heat-transfer pulse calculated as described above for a carbonaceous meteor and also for the same body using the method of ref. 1 (which assumed a uniform, constant shock layer temperature of 25,000 K). Our calculations yield temperatures which for most cases are below 20,000 K in the stagnation region and rapidly decrease towards the edge of the body because of the smaller shock angle. As a result of these lower temperatures and our analysis of the non-adiabatic effects and radiation absorption, the current method yields much lower heating rates than those calculated in refs 1, 2.

1. Chyba, C. F., Thomas, P. J. & Zahnle, K. J. *Nature* **361**, 40–44 (1993).
2. Sekanina, Z. *Astr. J.* **88**, 1382–1414 (1983).
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Although previous studies suggest that carbonaceous bodies would probably have released their energy too high in the atmosphere to have been responsible for Tunguska, our work, because of the more realistic, lower drag coefficient and the less severe calculated heating loads, indicates that a given meteor would airburst at a significantly lower altitude than previously believed^{1,2}. As a result, carbonaceous chondrites 50–100 m in diameter are found to airburst in the 6–10-km range characteristic of the Tunguska object.

Because these are the most common type of meteor to enter the Earth's atmosphere, they must be considered the most probable cause of the Tunguska event.

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Tuned directionality in cricket ears

SIR – Crickets (*Gryllus bimaculatus*) listening to the 4.5-kHz calling song have excellent directional hearing: the tympanal vibrations vary much with the direction of sound incidence^{1,2}, but the amplitude of sound pressure at the ears is almost constant³. The reasons for this apparent paradox are that sound reaches both the outer surface of the tympanum and the inner surface (through tracheal tubes from the ipsi- and contralateral thoracic spiracles, respectively; see Fig. 1a), and that the relative phases of the three sounds depend on the angle of sound incidence.

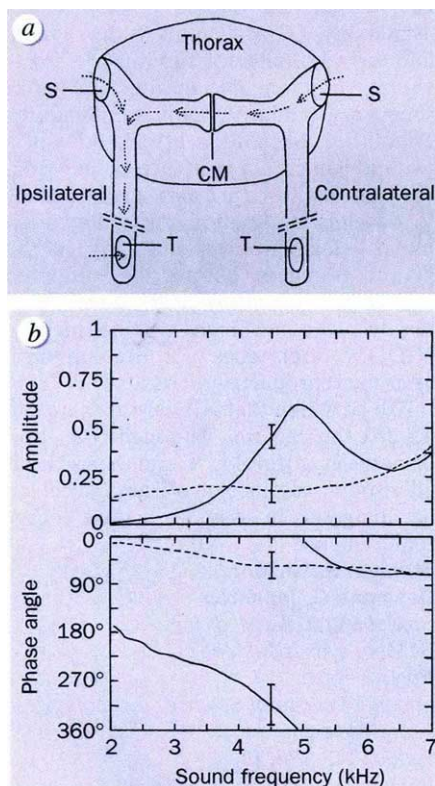


FIG. 1 a, The cricket hearing organ. CM, central membrane; S, spiracle (opening on thorax); T, tympanum (in front leg). Dotted arrows, sound paths. b, Amplitude and phase of sound arriving at the inner surface of a tympanum from the contralateral spiracle. Solid line, intact; broken line, perforated central membrane. The sound at the spiracle is set to an amplitude of 1 and a phase of 0°. Standard deviations indicated at 4.5 kHz ($n = 5$).

The transmission of sound from a spiracle to the tympanum can be studied by using the tympanal vibrations (recorded with laser vibrometry) for measuring the sound at the inner surface³. Sound from the contralateral spiracle is delayed by no less than 313° at 4.5 kHz when it reaches the tympanum (Fig. 1b, solid line). This is exactly right for the sound to be almost in phase with the (vectorial) sum of the other two sounds when the sound direction is ipsilateral, and almost out of phase for contralateral sound³. The result is the directional diagram in Fig. 2a (solid line).

The physical distance from the contralateral spiracle to the tympanum is much too small to account for the 313° delay, so some other factor must be responsible for the large delay. A central membrane (Fig. 1a) in the transverse trachea is involved. Holes of 10–25% of its area (made with the tip of a human hair) cause the phase at 4.5 kHz to drop to 54°, and a change is also seen in the amplitude of the transmitted sound (Fig. 1b, broken line). The result is a dramatic decrease in the directionality (broken line in Fig. 2a), previously observed using electrophysiological techniques⁴. Especially important for crickets walking towards singers is the disappearance of the gradient of auditory sensitivity in the forward direction (Fig. 2b, where 30° and 330° are typical extreme positions during phonotactic meandering).

In the intact animal, the change of phase varies much with frequency (Fig. 1b). A proper phase, which is a prerequisite for the high directionality of the ear, only exists within a narrow band around the calling song frequency (Fig. 2b). The mechanical phase shifting thus tunes the directionality of the ear.

Small animals living on the ground have a frequency band of only a few kilohertz available for long-distance communication. They are too small to emit low-frequency sounds, and high-frequency sounds suffer much attenuation and scattering⁵. They are thus forced to communicate at relatively low frequencies, at which directional hearing is difficult.

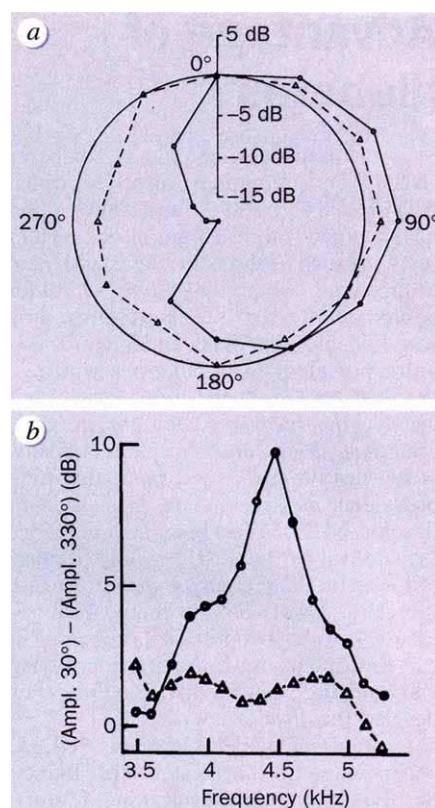


FIG. 2 a, Directional dependence of tympanal vibration at 4.5 kHz in a cricket with intact (solid line) and perforated central membrane (broken line). 0°, forward; 90°, ipsilateral sound direction. b, Difference between the tympanal vibrations at 30° and 330°. Solid and broken lines as in a.

Small grasshoppers with a pressure-difference hearing mechanism similar to that of crickets do not have a mechanical phase shifter in their interaural tracheal air sacs, and their directional hearing around 5 kHz is not very impressive⁶. Calculations show, however, that a much more useful directionality would exist if the sound arriving at the inner surface from the other ear had been more delayed.

The cricket thus stands out as an animal that has solved a major problem in auditory biophysics. It is tempting to speculate that the pure tone calling songs of crickets may have evolved as a consequence of the excellent directional hearing made possible within a very narrow frequency range by the mechanical phase shifter.

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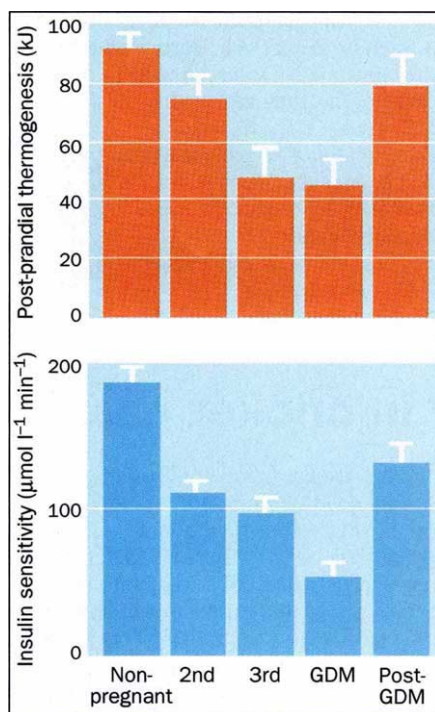
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Advantage of diabetes?

SIR — Non-insulin-dependent diabetes (NIDDM), a familial disorder which develops in middle and later life, has become more common in certain societies such as the Nauruan islanders in Micronesia, where 50–60% of older adults are affected (see the discussion by Jared Diamond in *News and Views*¹). It is more prevalent in populations which in the past have endured food scarcity but which now have a sedentary lifestyle, abundant food and frequent obesity. Neel's hypothesis² to explain this high prevalence is that the predisposition to develop NIDDM has been advantageous for survival in the past, possibly because of lower levels of energy expenditure and therefore lower dietary energy requirements. But this hypothesis has been difficult to test because it requires identifying and studying subjects at risk before they develop the disorder.

Gestational diabetes mellitus (GDM) occurs in the third trimester of pregnancy, but usually disappears following delivery. Most women with this condition develop impaired glucose tolerance or NIDDM in later life. The women are also likely to re-develop GDM in a subsequent pregnancy. We have studied post-prandial thermogenesis and resting energy expenditure in women with a previous pregnancy complicated by GDM, when they were pregnant again. They were studied in the second trimester, at which time glucose tolerance was still normal, and once more when glucose tolerance was normal following this later pregnancy. Pregnant women without a personal or family history of diabetes were studied for comparison. Details are reported elsewhere^{3,4}.

Post-prandial thermogenesis (also called *luxus consumption*, the specific dynamic action of protein and diet-induced thermogenesis) is the heat generated following the ingestion of food⁵, representing 14–20 per cent of total daily energy expenditure. In normal pregnancy (see figure), post-prandial thermogenesis decreases, resulting in an energy saving of 39 MJ over the second and third trimesters, or 13 per cent of the additional energy cost of pregnancy. In women with previous GDM, post-prandial thermogenesis decreases further, increasing the energy saving to 22 per cent. Post-prandial thermogenesis remains lower in women with previous GDM than in the women who had not had diabetes during pregnancy. In the non-pregnant state this energy saving amounts to 70 kJ per day. Resting energy expenditure increases as expected during pregnancy, but no differences are observed as a result of the diabetic predisposition.



Post-prandial thermogenesis (top) and insulin sensitivity (bottom) (mean s.e.m.) in non-pregnant women, in women during the second (2nd) and third (3rd) trimesters of normal pregnancy, in gestational diabetic women (GDM) studied in the second trimester (before decompensation to diabetes) and in women with GDM studied after delivery when glucose tolerance had returned to normal (post-GDM). Post-prandial thermogenesis was measured by continuous indirect calorimetry following the ingestion of a mixed meal (42 kJ per kg lean body mass). The integrated area of the increment in energy expenditure over resting energy expenditure was calculated and expressed in kJ. Insulin sensitivity was measured with a short insulin tolerance test. The linear slope of the arterialized plasma glucose decline after 0.05 U per kg body weight intravenous insulin (3–15 min) was calculated and expressed in $\mu\text{mol l}^{-1} \text{min}^{-1}$. Results for post-prandial thermogenesis were $P < 0.05$ both in GDM compared with 2nd trimester women and in post-GDM compared with non-pregnant women, while respective results for insulin sensitivity were $P < 0.01$. The data are modified, with additional data, from refs 3 and 4.

Post-prandial thermogenesis has an obligatory component (reflecting the energy costs of the digestion, absorption, interconversions and storage of food) and a possible facultative component (which has been proposed to modulate energy expenditure). Insulin action is thought to be important in its production. Thus, thermogenesis after eating is reduced in cafeteria-fed diabetic rats and is restored by insulin treatment⁶. In humans, thermogenesis in response to oral glucose is reduced in insulin-resistant states such as obesity, and is reduced further when obese subjects have impaired glucose tolerance⁷. The thermogenic response to insulin and

glucose given intravenously is also reduced in obesity and reduced further when insulin sensitivity is lower still in obese subjects with impaired glucose tolerance or diabetes mellitus⁸.

We found that normal pregnancy is associated with a decline in insulin sensitivity (see figure). In this situation, insulin sensitivity correlates positively with post-prandial thermogenesis, again suggesting that these parameters are linked. In the women with previous GDM, insulin sensitivity is reduced further and the reduction persists after delivery. Insulin sensitivity is impaired in other subjects predisposed to NIDDM, such as first-degree relatives of NIDDM patients⁹ (30–40 per cent lifetime risk), although their thermogenesis has not been studied.

The dietary energy saving associated with this insulin resistance in both pregnancy and the non-pregnant state is the evolutionary advantage proposed in this extension of the Neel hypothesis. No advantage is implied once GDM or NIDDM have developed. On the contrary, GDM has disadvantages resulting from fetal macrosomia. Dietary energy restriction is the mainstay of treatment and is sufficient in itself in most women. In situations of limited dietary energy availability, GDM is unlikely to develop and the advantage for survival of a lower energy requirement during and after pregnancy would predominate. Similarly, established NIDDM is associated with a twofold increase in mortality, mainly from vascular disease¹⁰, but also it is less likely to develop in situations of limited food intake. (Diabetes was unknown in the Nauru islanders before they adopted a Western lifestyle.) The evolutionary disadvantage may in any case be small, as NIDDM often does not develop until after the reproductive years.

We conclude that NIDDM is therefore adverse to health, although the predisposition to develop it, and the associated insulin resistance, may once have been advantageous in terms of dietary energy requirements.

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A dose of Dr Darwin

Melvin Konner

Why We Get Sick: The New Science of Darwinian Medicine*. By Randolph M. Nesse and George C. Williams. *Times Books/Weidenfeld and Nicolson*: 1995. Pp. 291. \$24, £20.

WHERE I live, a few hundred metres from the US Centers for Disease Control (CDC), I opened the local paper recently to find news of their report that in Atlanta 27 per cent of the cases of *Streptococcus pneumoniae* are resistant to penicillin, an order of magnitude higher than the national rate. Why the difference? No clue, really. A few days later, a team of spacesuit-clad CDC virologists sped to Zaire to examine a so far punctate explosion of Ebola, a horribly destructive virus that helped to inspire the recent movie *Outbreak*. If this seems a case of life imitating art, it is only one of many in the offing.

What these two unpleasant pieces of news have in common is a new consciousness of how infectious organisms work. It could have come many decades ago, if physicians and medical scientists had understood the simple ideas developed in this lively, informative book. The 'new' consciousness sees microbes as evolving. With the aid of what doctors call the retrospectroscope, it seems obvious. Indeed, if Paul Ehrlich, Robert Koch, Alexander Fleming and other early explorers in the jungle of infectious disease had packed a copy of Darwin, their nightly reading of him might have changed the course of history and prevented much unnecessary suffering. Because of course, as we see today, bacteria and viruses are trying to make a living like the rest of us, and when they stumble upon a new way of doing that — moving to humans from other primates, say, with a change in virulence, or degrading penicillinase, or resisting AZT — they are going to keep doing it. And as natural selection shapes the odds among them, they are going to do it better and better.

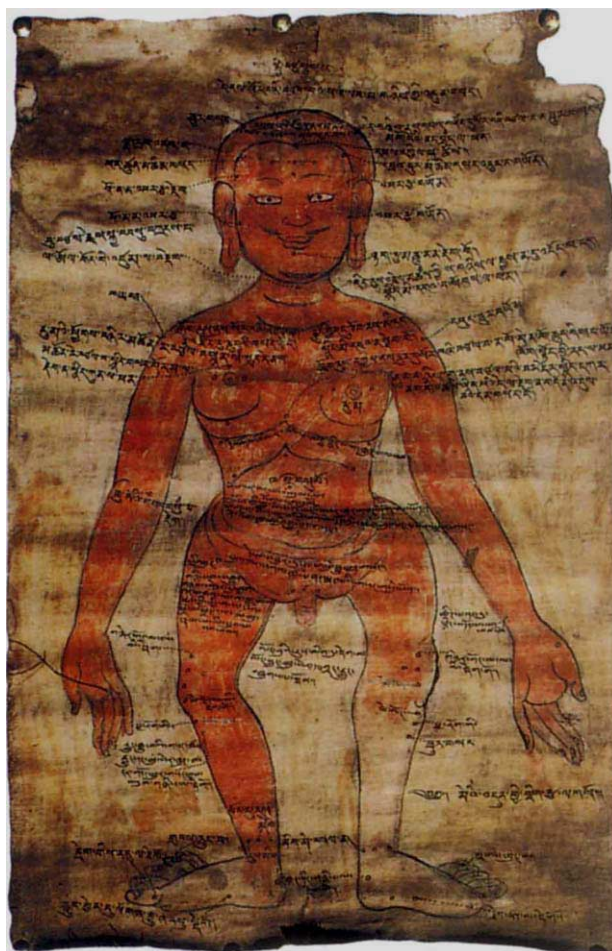
The shame of it is that if medical students had been made to study Darwin — and remember, they could have started in 1859 — the healing professions would have long since been imbued with a knowledge of nature that might have made a difference. The irresponsibility with which penicillin was distributed throughout the mid-twentieth century was not just likely to produce resistant organisms, it was certain to do so. Failure to see that was very like the failure, a century earlier, to notice that childbed fever was being spread by obstetricians — a decades-long lag in the adoption and application of a principle of science.

As for our new vigilance regarding emerging viruses, well, any evolutionist can tell you that the microbes have barely begun to fight, while we have barely begun to take their evolutionary dynamics seriously. A truly Darwinian epidemiology would be looking for new and newly virulent viruses behind almost every felled tree in the shrinking tropical forest. It is humbling to wonder what might have happened if we had had an early-warning surveillance apparatus in place to notice adaptive trends in the immunodeficiency viruses when they were just starting to be on the move evolutionarily. In this Darwinian 'arms race', with our current level of vigilance based on a pre-Darwinian consciousness, we are likely to be surprised again and again.

Randolph Nesse, a physician who teaches at the University of Michigan Medical School, and George Williams, a

professor at the State University of New York and one of the leading evolutionary theorists of our time, have written their book in an effort to prevent more such surprises. It does not have great scholarly or scientific depth, but it is intended for a general audience, not a scientific one. It is a needed preventive measure against the enduring, endemic nineteenth-century consciousness about nature that pervades medical thinking near the turn of the twenty-first century — a vaccine, one might say, against the lulling delusions of pre-Darwinian thought. Although Nesse and Williams did not originate the field of evolutionary medicine, they have helped to integrate and advance it, and with this volume they give both physicians and lay readers a chance to remedy, quite painlessly, the ignorance of evolution that expensive educations have usually left them with.

Resistant bacteria or parasites and 'hot' emerging viruses are only the most dramatic puzzles that evolutionary medicine could have helped us solve sooner. Would post-war paediatricians, at the peak of their persuasiveness, have made a blanket recommendation against breast feeding if they had had the slightest evolutionary perspective? Laborious exploration of the immunological and



DRAWING from a Tibetan medicine manual showing the placement of 'fire tips', hot iron rods, used in the treatment of injury. The picture is taken from *Caravans of the Himalaya* by Eric Valli and Diane Summers. The authors spent two years with the Dolpo-pa, a nomadic people who live between the high peaks of the Himalaya and the Tibetan plateau Chang Tang. With the help of many outstanding colour photographs, they recount their adventures among this fascinating civilization while exploring its history and traditions. Thames and Hudson, £36.

* UK title: *Evolution and Healing*.

anti-allergenic properties of human milk has been of value, to be sure, but a knowledge of the functional history of lactation could have saved women and babies a lot of pain while these explorations were under way. Would the obstetricians of the 1950s have recommended 'twilight sleep' as the best way for a woman to go through childbirth, reluctantly acknowledging the 'naturalness' of birth only after two decades of patients' demands, if they had seen human delivery in its evolutionary context?

The consciousness now forming about chronic mid-to-late-life degenerative disease is bringing medical thinking into line with another kind of evolutionary reality. These diseases — atherosclerotic cardiovascular disease, diabetes, alcoholism, hypertension and several common cancers — must be increasingly thought of as 'diseases of civilization'. They are the great sources of morbidity and mortality in the industrialized world; rare or unusual in the kinds of societies in which we spent most of human history (even after correcting for age and the impact of infections); and often demonstrably the result of our pervasive, ingrained and yet quite novel living habits. In evolutionary terms, there is a discordance between the genome evolved in adaptation to a radically different environment and the new metabolic conditions in which we have now steeped those genes. Not surprisingly, they have not adapted to this sudden change.

I knew that medical consciousness was changing when I heard a renowned physician-scientist, an authority on the genetics of lipid disorders, say in a lecture that it is not "natural" for humans to have cholesterol levels in the mid-200s (milligrams per decilitre). But he went on to cite average levels in Japan as his evidence. American-Japanese differences are of interest, but the evolutionary perspective demands a much broader look at different types of human societies. Such a look strongly suggests that a serum cholesterol of 190 is not natural either, and that we may some day be targeting the mid-100s.

Of course, no mere theory, even bolstered by cross-population data, can clinch a piece of clinical advice; there is evidence that low cholesterol, for example, may have a medical downside. But theory can suggest randomized controlled trials and laboratory experiments that otherwise might not be done. Could truly low salt intake — less than a gram a day — prevent hypertension even if there is no dose response in a higher range? Could letting a fever, within limits, take its natural course make life more difficult for certain common bacteria? Could too many ovulatory cycles be the key to understanding major gynaecological cancers? Could the hormones in human milk help lull a baby to sleep? None of

these questions can be answered by evolutionary analysis, but each of them is strongly suggested by such analysis.

What Nesse and Williams, along with S. Boyd Eaton, Matthew Kluger, Paul Ewald and others in this exciting field, are trying to tell us is that evolutionary theorizing points to hypotheses that we otherwise might not even think of. They are not trying to substitute theory for experiment, but to accelerate the process by which experiment leads to clinical knowledge. The preventable mistakes of the past, due to ignorance of evolution — sometimes pig-headed and 'principled' but usually just lazy — should suffice to convince us that they are right.

But perhaps even more important than the suggestion of specific hypotheses is the change in consciousness that medical scientists need. The Bohr model of the atom did not change workaday chemistry overnight, and it has been a long haul from the identification of the structure of DNA to the methods that discovery now brings to the street-corner clinic. Subtler still is the way in which quantum theory has slowly but surely pervaded and altered practical physics. But in the case of evolution the delay has already been inexcusably long. It is high time Hippocrates gave Darwin his due. □

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Whither medicine?

Stephen Lock

Science and the Quiet Art: The Role of Medical Research in Health Care*. By David Weatherall. Norton/Oxford University Press: 1995. Pp. 378. \$25, £17.99.

"WE'VE paid for 45 years of discovery: let's start requiring its application to the critical problems in the civilian sector." In *Science and the Quiet Art*, David Weatherall answers this recent plaint by a US congressman by showing that, in medicine, solutions are not so easy: practical developments take a long time and cannot be bespoke. More than 60 years elapsed, after all, between Koch's discovery of the tubercle bacillus and Waksman's development of streptomycin, which did so much to relieve human misery, let alone the strain on the health services. Major discoveries, Weatherall shows, do not come to order: rather, they arise out of curiosity-driven research, usually in centres of excellence, when fresh knowledge in unrelated and often new disciplines suddenly comes

together and can then be applied by talented doctors. Nevertheless, modern medicine will not achieve its full potential until we are all more scientifically literate.

As a fellow of the Royal Society, London, and regius professor of medicine at the University of Oxford, Weatherall would say all this, wouldn't he? But, as well as being a haematologist and molecular biologist, he is also a humane clinician as preoccupied with the ethical implications of discoveries as with making them, and with the problems of the developing as well as the developed countries. Moreover, unlike many doctors, Weatherall is also sympathetic to the greens and the 'medical sceptics' — Ivan Illich, Thomas McKeown and Archie Cochrane — who argued that improvements in health have had little to do with medical discoveries: rather, the improvements reflect greater prosperity, with more food and better bathrooms. His book, then, commissioned by the Commonwealth Fund, aims at teasing out all these strands for the general reader, looking at the successes and the failures of modern scientific medicine and predicting where it may be leading us.

Using cardiology to illustrate the evolution of a specialty, Weatherall charts its beginnings from clinical observations (including the invention of the stethoscope), through the correlation between pathology and physiology (coronary thrombosis and electrocardiographic abnormality) and catheterization and direct radiographic visualization of the heart, to today's non-invasive methods using magnetic resonance and positron emission tomography. Complementing such advances have been new drugs effective against heart failure, hypertension and thrombosis, as well as surgery to correct abnormalities of the heart valves and blood vessels, or, ultimately, transplantation (itself not feasible until basic research in immunology had shown how to overcome graft rejection).

Despite such advances, however, Weatherall concludes that cardiology is still an uneasy mixture of solid science and unproved practice. Doctors have reached an impasse in understanding the principal killers, such as coronaries and strokes, perhaps, he believes, because they have mistakenly tried to replicate their successes against one particular single-cause killer, the infections. The origins of the other killers, however, are multifactorial, particularly a genetic make-up inappropriate to today's environment; genes useful in the past — protecting against, say, malaria — have become deleterious today. Progress is therefore likely to be slow and, anyhow, a good case can be made that humankind is programmed to live to 85, so epidemiology and modern advances should aim

* UK subtitle: *Medical Research and Patient Care*.

at ensuring that we get there, and in comfort. Nevertheless, I wonder if Weatherall is not over-optimistic; given epidemiology's discovery of one megakiller, no society has yet removed it. Distressingly, also, younger people are ignoring the message that cigarettes kill: the activists who recently occupied an oil rig were apparently an intelligent but puritan lot, except that they smoked like chimneys.

Perhaps, though, such optimism reflects an ultimate failure of conviction on the author's part. For, although concluding that the medical sciences are moving into their most exciting and productive period, Weatherall seems suddenly to lose his nerve. To be sure, molecular biology and genetics will soon dominate human biology and medicine, but any benefits for the patient are uncertain and will, he thinks, take a long time. So he returns to talking about the art as well as the science, remembering that one of his teachers used "the quiet art", a quotation from Virgil, as the title for an anthology about medical practice. In the end we are left confused.

Part of the uncertainty arises, I believe, because the author himself was divided about his book's message — a polemic urging decision-makers to support non-targeted research, a popular exposition, or both? Moreover, the reader faces another difficulty: its length. On the one hand, Weatherall's details give a marvellous overview of the medical sciences at a turning point, the sweep recalling such masterly syntheses for the general reader as Lancelot Hogben's *Mathematics for the Millions*, say, or H. G. Wells's *An Outline of History*.

On the other hand, one has to echo the Austrian emperor on *La Clemenza di Tito*: "Too many notes, my dear Mozart!" Weatherall usually writes crisply and stylishly, but in these 348 pages there is a book of 100 pages struggling to get out. He acknowledges his secretary's help in transcribing his scrawl, but I suspect that (as busy men do) he dictated most of it into a tape recorder without alteration. He has been ill-served by his editors: creative editors still exist and would have excised the superfluous — the heavy use of adjectives and adverbs, the predilection for sentences beginning with 'and' or 'but', and for the word 'seminal', as well as the academic asides and chuckles together with the repetitions (heart block and introns and exons, for example, both explained again within ten pages). This book is too important to be buried; somebody should lick it into shape. □

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States of mind

Karl J. Friston

Dynamic Patterns: The Self-Organization of Brain and Behaviour. By J. A. Scott Kelso. MIT Press: 1995. Pp. 334. \$49.95, £44.95

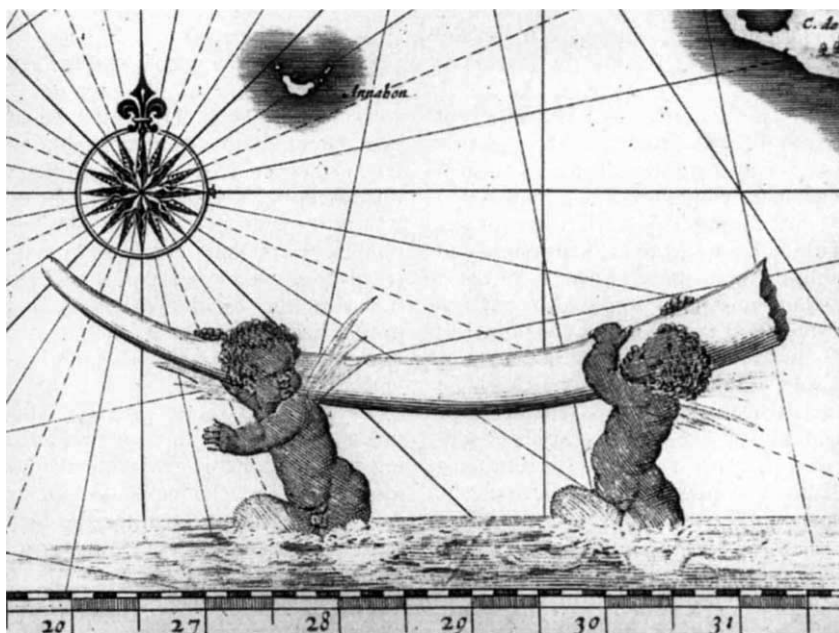
THIS charming and thought-provoking book looks at the brain and behaviour as pattern-forming dynamical systems. It captures, and gives substance to, a new theme in neuroscience, in which complexity is in the ascendancy and good old-fashioned chaos seems a thing of the past. Indeed, it is now almost *passé* to point out that complex biological systems are chaotic.

Complexity is brought sharply into focus when considering the relationship between functional segregation and functional integration of brain dynamics. Biological systems are found near phase-transitions, in regimes of metastability or at 'the edge of chaos'. This theme appears repeatedly in the sciences of complexity and evolutionary theory and (in this book) in the coordination dynamics of self-organizing systems.

Central to Kelso's account is the role of critical points (regions of parameter space where the attractor changes qualitatively). Systems near critical points, or instabilities, show characteristic behaviours, such as critical slowing and critical fluctuations, phenomena expressed by the system's order parameters — collective variables that embody the essential dynamics. Other

people have reached a similar conclusion: Stuart Kauffman's self-organizing systems are found between low-order attractors and chaotic regimes of parameter space. Gerald Edelman and colleagues see a necessary balance between a state of globally coherent integration and disintegrated functional segregation, where each brain area preserves its distinct dynamics. For Kelso the key to self-organization is instability, where the system is near crisis as it approaches a critical point. Self-organization is simply the expression of dynamical patterns typical of nonlinear systems near critical points. These systems are generally characterized by loose couplings among their order parameters. Kelso frames this informational coupling in terms of coordination dynamics and, inspired by synergistics, the resulting dynamical features. He carefully explains all these technical terms and demystifies the concept of self-organization with a series of lucid examples, starting with a pan of boiling oil in the kitchen with his daughter and ending with a spatial-mode analysis of multi-channel magnetoencephalography data.

The book falls into three parts. After presenting a general and accessible account of dynamic pattern formation in simple nonlinear systems, Kelso demonstrates the same principles and phenomena in human sensorimotor coordination. He introduces the Haken-Kelso-Bunz model, which becomes an old friend as the book unfolds. The first part concludes by relating relative coordination (the incomplete entrainment of coupled systems,



Ivory first began to be extensively exploited in the seventeenth century, when the Dutch and English established themselves in Sierra Leone. This early Dutch map of Guinea reveals the European agenda. It is one of hundreds of pictures reproduced in *Elephant: The Animal and Its Ivory in African Culture* edited by D. H. Ross, which arose from an exhibition held in 1992 at the Fowler Museum of Cultural History, Los Angeles. University of California Press, \$49 (pbk).

such as a father and son walking on a beach or two neurons in the extrastriate cortex) and intermittency in nonlinear dynamical systems. Kelso is clearly proud of this insight — many of his original ideas pre-date the comfortable jargon (and mathematical formalism) of nonlinear dynamics. The second part of the book deals with intention, learning and perception. An interesting notion is that intentionality is meaningful only in terms of the system's order parameters. This precludes intention as a concept separable from the system enacting that intention. There is a seductive internal consistency here, but there is also a sense that some deep teleological question has been sidelined. Plasticity learning and memory are framed in terms of changes in the attractor landscape; although this is not as revolutionary as Kelso seems to think, his formulation of these changes in the language of coordination dynamics and synergistics is compelling. The final part addresses the brain as a self-organizing (pattern-forming) system using earlier themes as well as explicit measures of brain activity such as magnetoencephalography.

Is Kelso's account of brain and behaviour complete? It confronts the reader with many fundamental issues, such as the principles of pattern formation, the central role of instabilities and crisis in nonlinear systems, the brain as a self-organizing system, the fallacy of the computer-program metaphor, circular causality (where influences among different scales transcend notions such as top-down processing or reductionism) and learning as a plastic reshaping of the attractor manifold (where the learner is as important as what is learned). A mechanistic understanding is not one of Kelso's primary concerns, so the theory is a little incomplete when it comes to such questions as why the brain is poised in a state of metastability or what brings intentional systems to critical points and maintains them there. Answers to these questions have been proposed by authors referred to in the book but are not dealt with here in any great depth. For example, metastable systems may be more evolvable and, by selection for selectability, come to be a dominant phenotypic feature (such as Kauffman's "selective meta-dynamics"). Alternatively, metastability may be necessary for adaptive behaviour. Metastability itself could even be subject to selective pressure, with genetic mechanisms promoting it (such as Edelman's "value"). Whatever, Kelso's book will get you thinking. □

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Constructing reality

Tony Sudbery

Schrödinger's Kittens and the Search for Reality. By John Gribbin. Weidenfeld and Nicolson/Little, Brown: 1995. Pp. 261. £18.99, \$23.95.

In *In Search of Schrödinger's Cat*, his previous popular book on quantum mechanics, John Gribbin emphasized the empirical successes of this strangest of physical theories and read the lesson that we must be prepared to accept its incomprehensibility and be grateful that it works so well. In this sequel he is less severe, promising an account of new work that successfully dispels the mystery and paradox of quantum mechanics.

This is exciting news. To make it even more exciting, the mysteries of quantum mechanics have deepened and strengthened in the ten years since Gribbin wrote his earlier book. Advances in experimental techniques have brought many quantum effects out of theory textbooks and into the laboratory; thought experiments have become actuality, and paradoxes have become effects. Quantum jumps can now be seen (with the naked eye) in a single atom; the two-slit experiment is a genuine laboratory experiment; and the quantum-mechanical theorem that repeated observation of a system freezes its development ("a watched pot never boils") has been verified by real observations of trapped atoms.

Gribbin describes some of these experimental developments (although not, I am sorry to say, Hans Dehmelt's beautiful experiment on quantum jumps) and some recent theoretical ideas such as quantum cryptography, after a historical introduction that traces, in particular, the history of theories of light. But then comes the problem of making sense of it all. Gribbin describes the usual approaches to this problem. His attitude, which many would share, is summarized by his chapter title: "Desperate Remedies".

He is, on the whole, a reliable teller of this story, although there are disconcerting moments when a mad glint comes into his eye as he thinks of the amazing denouement he has in store. The logic of his narrative, however, is weak. There are several points at which an uninformed reader would be lost, possibly without realizing it — for example, in the story of the 'EPR paradox', David Bohm and John Bell. Particularly on matters of interpretation, I find Gribbin often confused or superficial or both: witness his description of recombining universes as a "neat development beyond Everett's original idea" when it is clearly implicit in Everett's

paper, or his apparent bafflement at David Deutsch's common-sense discussion of time. But the greatest confusion comes in the penultimate chapter, where Gribbin turns to the sociology of knowledge.

One of my favourite pieces of unconscious humour is the following quotation from Andrew Pickering's *Constructing Quarks*: "It is *unproblematic* that scientists produce accounts of the world that they find comprehensible: given their cultural resources, only singular incompetence could have prevented members of the [physics] community producing an understandable version of reality at any point in their history."

Gribbin quotes this passage in order to endorse it, even though he has just devoted a chapter to physicists' *failure* to produce an understandable version of reality. He proceeds to the idealist conclusion that "[r]eality is in very large measure what you want it to be". (I must get him to tell me what I'm doing wrong.)

He now finds himself, at the opening of his final chapter, in an awkward position. Having lured us on throughout the book with the promise of the one true answer at last, he finds that he has just admitted that his goods are worthless. Somewhat feebly, and with the air of someone who has written all this stuff and is damned if he's going to waste it, he gives us the answer anyway. This is John Cramer's transactional interpretation, which describes a negotiation between emitter and absorber, the latter sending its signals backwards in time. We are then left to decide whether this kind of market is what (in very large measure) we want reality to be. □

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New in paperback

The Collapse of Chaos: Discovering Simplicity in a Complex World by Jack Cohen and Ian Stewart. Penguin, £8.99. In a review in *Nature*, John L. Casti wrote: "A host of new and thought-provoking ideas about the workings of complex systems. . . as good an introduction to the science of the future as any you're likely to find".

The Stuff of the Universe: Dark Matter, Mankind and Anthropic Cosmology by John Gribbin and Martin Rees. Penguin, £7.99. A journalist teams up with an internationally renowned astronomer in a discussion of the relationship between humankind and the cosmos. Originally published as *Cosmic Coincidences* in 1990.

Atoms in the Family: My Life With Enrico Fermi by Laura Fermi. University of Chicago Press, \$15.95, £11.25. A classic account of the career of one of the world's great atomic physicists, written in 1954 by his wife.

The synaptic vesicle cycle: a cascade of protein-protein interactions

Thomas C. Südhof

The synaptic vesicle cycle at the nerve terminal consists of vesicle exocytosis with neurotransmitter release, endocytosis of empty vesicles, and regeneration of fresh vesicles. Of all cellular transport pathways, the synaptic vesicle cycle is the fastest and the most tightly regulated. A convergence of results now allows formulation of molecular models for key steps of the cycle. These developments may form the basis for a mechanistic understanding of higher neural function.

SYNAPTIC transmission is the currency of information exchange in the brain. Billions of neurons each form thousands of synapses, each of which in turn is independently regulated. The resulting complexity vastly surpasses, for example, the complexity of the human genome. From this apparent chaos emerges the pristine order of information processing by the brain. The organizing principles that govern synaptic transmission are now coming into view.

The key event in the synaptic vesicle (SV) cycle is exocytosis by membrane fusion. Membrane fusion occurs by a similar mechanism in organisms from yeast to man, and in organelles from the nuclear envelope to SVs^{1,2}. The special needs of a synapse, however, create unique requirements. Synaptic transmission requires a localized and rapid signal that can be repeated at high frequency and up- or downregulated with time³. These needs are satisfied by three unique events in the cycle: (1) before exocytosis, SVs are predocked at a specialized area of the presynaptic plasma membrane called the active zone; (2) the probability that a single docked SV will undergo exocytosis in response to Ca^{2+} is low and can be modulated over a wide range; and (3) after exocytosis, SVs quickly undergo endocytosis and recycle locally close to the site of exocytosis.

These properties require a level of control over the fusion process that is more complex than is observed in other fusion events. Insight into the special features of the SV cycle have recently been gained from four major approaches: (1) study of synaptic protein-protein interactions; (2) study of mechanisms by which botulinum and tetanus toxins inhibit neurotransmitter release; (3) study of invertebrate preparations with disrupted SV traffic; and (4) study of mutations in mice induced by the 'gene knockout' technology. The current review explores the implications of these studies for the molecular steps underlying SV traffic.

The synaptic vesicle cycle

The SV cycle can be divided into 9 steps (Fig. 1).

(1) Docking. SVs that are filled with neurotransmitters dock at the active zone. Docking, defined as the initial contact between the membranes, occurs only at the active zone opposite to the synaptic cleft, implicating a targeting process.

(2) Priming. After docking, SVs go through a maturation process that makes them competent for fast Ca^{2+} -triggered membrane fusion. Such a priming step is suggested by three observations: first, the speed of Ca^{2+} -triggered SV exocytosis which is too fast for a complex multistep reaction⁴; second, most of the docked SVs cannot be triggered to fuse by Ca^{2+} immediately and therefore appear to be not yet competent to fuse^{5,7}; and third, during extensive repetitive stimulation, SV exocytosis slows down before the number of docked SVs declines⁸. These results suggest that docking is not rate limiting and that SVs are not immediately competent to fuse after dock-

ing and need to go through a rate-limiting priming step. Priming may initiate exocytosis by executing a partial fusion process. At this point SVs arrest, awaiting a Ca^{2+} signal.

(3) Fusion/exocytosis. Primed SVs are stimulated for rapid fusion/exocytosis by a Ca^{2+} spike during an action potential. Although Ca^{2+} -triggered exocytosis requires less than 0.3 ms (ref. 4), Ca^{2+} triggering is inefficient: only one of many docked SVs fuses, and only one in every three to ten action potentials leads to exocytosis^{5,7}.

(4) Endocytosis. Empty SV membranes are rapidly internalized, probably by way of clathrin-coated pits which become coated vesicles⁹.

(5) Translocation. Coated vesicles shed their coats, acidify and translocate into the interior, becoming recycling SVs.

(6) Endosome fusion. Recycling SVs fuse with early endosomes, as suggested by the high content in SVs of rab5, an endosomal marker, and by the labelling of synaptic endosomes with soluble markers during stimulation^{9,10}.

(7) Budding. SVs are regenerated primarily by budding from endosomes.

(8) Neurotransmitter uptake. SVs accumulate neurotransmitters by active transport driven by an electrochemical gradient created by a proton pump.

(9) Translocation. SVs filled with neurotransmitters translocate back to the active zone either by diffusion or by a cytoskeleton-based transport process.

The entire SV cycle takes approximately 1 min^{11,12}. Exocytosis/fusion occurs in less than 1 ms, endocytosis in less than 5 s, and the remaining 55 s are used for the other steps of the cycle. The SV cycle resembles other transport pathways that shuttle membranes between the cell surface and endosomes, in particular receptor-mediated endocytosis. However, the SV cycle is unique in its speed and its tight regulation.

Selected properties of central synapses are summarized in Box 1. Although SVs are heterogeneous in size, there is no heterogeneity in function, as revealed by direct observation of recycling SVs using fluorescent dyes¹¹. The paucity of cytoskeletal elements close to the active zone indicates that SVs translocate by diffusion¹³, although the cytoskeleton could have an important regulatory role. In the nerve terminal, free SVs are linked into clusters by thin strands. The strands were originally thought to be composed of the SV protein synapsin I, but are also present in ribbon synapses which lack synapsins¹⁴, suggesting that they are made up of a different protein.

The entire SV cycle takes place locally in nerve terminals. This autonomy allows synapses to function at long distances from the cell body, a prerequisite for fast exocytosis and recycling. For example, the synapses of spinal cord neurons that innervate calf muscles are 1 m from the cell body, a distance that would take weeks to cover by organelle transport. The autonomy of nerve terminals makes it possible to regulate different synapses

BOX 1 Quantitative parameters of central synapses

Feature	Synapse type	Value
Nerve terminal volume (μm^3) ^{105,106}	Hippocampus*	0.11±0.11
	Cerebellum†	0.25±0.12
SVs per terminal ^{105,106}	Hippocampus*	223±245
	Cerebellum†	485±246
Synaptic contact area (as PSD area; μm^2) ^{105,106}	Hippocampus*	0.07±0.08
	Cerebellum†	0.15±0.08
Number of docked SVs per active zone ¹⁰⁷	Cortex	10–30
Number of readily releasable quanta (SVs) ¹⁰⁸	Hippocampus‡	12–24
SV diameter (nm) ^{105–107}	Hippocampus‡	34±3.8
	Cerebellum†	35±4.5
Synaptic cleft (nm) ¹⁰⁷	Inhibitory	13
	Excitatory	16
Release probability ^{5–7}		
High-probability terminals (15%)	Hippocampus* (slices)	0.37±0.04
Low-probability terminals (85%)		0.06±0.01
High-probability terminals	Hippocampus‡ (autapses)	0.54±0.05
Low-probability terminals		0.09±0.02
Ca ²⁺ requirement for release (μM) ⁶¹	Bipolar neurons§	~0.2 mM
Ca ²⁺ cooperativity ^{53,57}	Hippocampus‡	4
	Motoneurons	4
Synaptic delay between membrane depolarization and release ⁴	Motoneurons	~0.2 ms
Frequency of spontaneous release ('minis'; events per minute and terminal) ⁵⁷	Hippocampus‡	1–2
Time to replenish readily releasable quanta (priming) ¹⁰⁸	Hippocampus‡	~10 s
Kinetics of endocytosis ⁷¹	Bipolar neurons§	1.2–2.2 s
SV recycling time ¹²	Hippocampus‡	30–60 s

* Schaffer collateral/commissural fibre synapses onto pyramidal neurons in CA1.

† Parallel fibre synapses onto Purkinje cells.

‡ Synapses of isolated cultured pyramidal neurons from embryonic hippocampus.

§ Goldfish bipolar neuron ribbon synapses.

of the same neuron independently. This forms the basis for the selective modulation of subsets of synapses¹⁵, a process that integrates synaptic circuits.

The small size and simple protein composition of SVs has allowed their detailed characterization. The proteins of SVs can be divided into two functional classes: proteins involved in the uptake of neurotransmitters (transport proteins), and proteins that mediate SV membrane traffic, such as docking, fusion and budding (trafficking proteins)¹⁶. The structures of the major trafficking proteins of SVs are depicted in Fig. 2. A glossary of the proteins of SVs and their interacting partners at the synapse is given in Box 2. Major insights into the functions of synaptic proteins have been gained by studies of mutations and of toxins, which are summarized in Box 3. Although most SV proteins are specific for SVs, they contain no common motif that would suggest a sorting mechanism. Confusingly, but interestingly, SV proteins are present in multiple isoforms that are differentially expressed in subsets of neurons. No two protein families show the same distributions, and there is no correlation between their distributions and known functional differences of synapses.

Rab3 function in exocytosis

Rab proteins (rabs) are GTP-binding proteins of low molecular mass which direct membrane traffic^{17,18}. So far, more than 30 rabs have been identified. SVs contain at least two major rab families: rab3A and 3C, which are specific for SVs and secretory granules¹⁹, and rab5, which is required for endosome fusion in all cells²⁰. Rab3A is the most abundant rab protein in the brain, accounting for approximately 25% of its rab GTP-binding capacity²¹. Although most neurons express rab3A, a subpopulation of neurons also synthesize high levels of rab3C (ref. 22).

At the carboxy terminus, rab3 and other rabs contain two hydrophobic geranylgeranyl groups that tightly attach them to membranes. Nevertheless, a sizeable fraction of rab3 in brain is present in the cytosol, where it is complexed with GDI, an abundant protein that binds to most rabs in the GDP-bound

state²³. These findings suggest the model shown in Fig. 3: at rest, SVs contain GTP-bound rab3. When rab3 function is stimulated, its GTP is hydrolysed to GDP. GDP-rab3 is removed from the SV membrane by GDI, and rebound to new SVs under GDP-GTP exchange.

The tight regulation of membrane transport in the nerve terminal made it possible to test this model. Triggering of SV exocytosis led to the dissociation of rab3A and rab3C from SVs¹⁹ and dramatically increased the GDP:GTP ratio of rab3A (ref. 24). These findings indicated a role for rab3 in SV exocytosis, and it was surprising to find that rab3A knockout mice have no fundamental defects in SV exocytosis²¹. Synapses from rab3A-deficient mice released neurotransmitters normally when stimulated. However, when rab3A-deficient synapses were stimulated repetitively they exhibited profound synaptic depression. Thus rab3A is not essential for docking or fusion of SVs. However, rab3A is required to maintain a normal reserve of SVs for accelerated exocytosis during repetitive stimulation when SV recycling becomes rate limiting.

The action of rab3s during repetitive stimulation may be performed in collaboration with another SV protein, rabphilin-3A, which binds rab3A and rab3C in a GTP-dependent manner^{22,25}. In rab3A-deficient mice the levels of rabphilin-3A are decreased by more than 70%, but only in the synapses of neurons that do not make rab3C. In these neurons, rabphilin-3A remains in the cell bodies and is degraded, whereas neurons that contain rab3C transport rabphilin-3A to nerve terminals²². Thus a major function of rab3A and rab3C may be to recruit rabphilin-3A to SVs where rabphilin-3A could act as an effector for rab3s (Fig. 3).

Priming vesicles for fusion

A convergence of recent results from biochemical and genetic studies^{26–30} allows formulation of a model which, although incomplete, may approximate the molecular events in SV exocytosis (Fig. 4). According to this model, SVs dock and then proceed through a partial fusion reaction, priming, to make them

BOX 2 Glossary of synaptic proteins involved in neurotransmitter release

(1) SV proteins

Cysteine string proteins (CSPs). Peripheral membrane proteins containing more than 10 palmitoylated cysteines and a DNA-J homology domain⁸⁶.

Cytochrome b561. Electron transport protein required for intravesicular monooxygenases in subsets of SVs¹⁰⁹.

Neurotransmitter transporters. At least five types transport glutamate, acetylcholine, catecholamines, glycine/GABA and ATP. Catecholamine and acetylcholine transporters are homologous to each other and to bacterial drug-resistance transporters^{110,111}.

Rab and ral proteins. Rab3A, rab3C, rab5, rab7 and ral¹⁰⁹. Rab proteins cycle between cytosolic and membrane-bound forms, and not all SVs contain all rab proteins at the same time (Fig. 3).

Rabphilin-3A. Peripheral membrane protein that binds to rab3A and rab3C in a GTP-dependent manner, is phosphorylated by multiple kinases, and contains two C-terminal C₂-domains which may bind Ca²⁺ and phospholipids^{22,25}.

SCAMPs (Secretory carrier membrane proteins). Ubiquitous integral membrane proteins of secretory and transport vesicles¹⁰⁹.

SV2s. Highly glycosylated proteins with at least three isoforms (SV2A, B and C) containing 12 transmembrane regions and homology to bacterial and eukaryotic transporters^{2,109}.

Synapsins Ia, Ib, IIa and IIb. Monotopic membrane proteins that share N-terminal domains, including phosphorylation sites for CaM KI and PKA, but diverge C-terminally. Synapsins Ia/b have C-terminal phosphorylation sites for CaM KII and CDK V, and bind to actin microfilaments, microtubules, SH3-domains, calmodulin and annexin VI^{92,100}.

Synaptobrevins (VAMPs). Short type-2 membrane proteins that are cleaved by tetanus toxin and by botulinum toxins B, D, F and G^{44,45}.

Synaptogyrin. Polytopic membrane protein that is tyrosine-phosphorylated (K. Stenius, R. Janz, T.C.S. and R. Jahn, submitted).

Synaptophysins. Polytopic membrane proteins including synaptophysin that are tyrosine-phosphorylated and bind to synaptobrevins¹⁰⁹.

Synaptotagmins. Membrane glycoproteins with at least eight isoforms that bind Ca²⁺ and phospholipids and interact with neurexins, AP2 and syntaxins^{64,65,84}.

Transport proteins (channels) for chloride and zinc. Components of SVs postulated by the high zinc content of some SVs and the requirement of chloride flux for glutamate uptake¹⁰⁹.

Vacuolar proton pump. Protein complex of more than 15 subunits that constitutes the largest component of SVs¹⁰⁹. Establishes electrochemical gradient for neurotransmitter uptake.

(2) Proteins that associate with SVs

Amphiphysin. Nerve-terminal protein that associates with SVs probably via AP2 bound to synaptotagmin^{85,109}.

AP2 and clathrin. AP2 is a protein complex that binds to a specific

receptor on SVs and plasma membranes to trigger assembly of clathrin for endocytosis.

Ca²⁺, calmodulin-dependent protein kinases I and II (CaM KI and KII). These may transiently associate with SVs to phosphorylate synapsins and rabphilin-3A.

Dynamin 1. GTPase required for endocytosis that is phosphorylated by PKC, dephosphorylated by calcineurin upon membrane depolarization, and binds to AP2 (refs 78–81).

Dynein. Motor protein mediating microtubule-based SV transport, possibly retrograde axonal transport to the cell body¹¹².

GDP-dissociation inhibitors (GDIs). Bind isoprenylated rab proteins in the GDP-bound form, resulting in a cytoplasmic complex²³.

Kinesins. Motor proteins for microtubule-based SV transport. In *C. elegans*, a kinesin encoded by *unc-104* is essential for transport of SVs to nerve terminals¹¹³.

MSS4. Ubiquitous protein that tightly binds to a subgroup of rab proteins including rab1, rab3 and rab8 (ref. 114).

pp60^{src}. Tyrosine kinase that phosphorylates synaptophysin and synaptogyrin¹⁰⁹.

(3) Synaptic plasma membrane proteins

Munc13s. Mammalian homologues (13-1, 13-2 and 13-3) of the *C. elegans unc-13* gene which is essential for exocytosis¹⁰⁵.

Neurexins. Nerve-terminal cell-surface proteins with more than 1,000 isoforms generated by alternative splicing for three genes. Neurexins include the receptor for α -latrotoxin and may function in cell-cell recognition in the nervous system⁶⁶.

SNAP-25. Abundant peripheral membrane protein that is palmitoylated, cleaved by botulinum toxins A and E, and binds to syntaxins^{44,45}.

Syntaxins (originally named HPC-1). Ubiquitous type-2 membrane proteins that are cleaved by botulinum toxin C1 and bind to synaptotagmins, SNAP-25, synaptobrevins, SNAPs, Ca²⁺ channels and munc18s^{44,45}.

Voltage-gated Ca²⁺ channels. Concentrated at the active zone where their activation results in microdomains of high Ca²⁺. Several distinct types are used at synapses. N-type Ca²⁺ channels bind to syntaxin 1A¹¹⁶.

(4) Proteins that reversibly associate with plasma membrane proteins

Complexins. Nerve-terminal syntaxin-binding proteins (H. McMahon, M. Missler and T.C.S., unpublished data).

Munc18s. Mammalian homologues of the *C. elegans unc-18* gene and the *secl*, *slyl* and *slpl* products of yeast. They bind tightly to syntaxins^{34–38}.

N-ethylmaleimide sensitive factor (NSF). Trimeric ATPase required for *in vitro* membrane fusion during vesicular transport⁴².

$\alpha/\beta/\gamma$ -SNAPs. Soluble NSF-attachment proteins required to recruit NSF to membranes in an ATP-dependent manner⁴³.

competent for the final Ca²⁺-triggered step. During priming, a complex called the core complex is assembled by three abundant synaptic proteins, two from the plasma membrane (syntaxin and SNAP-25) and one from SVs (synaptobrevin/VAMP). The core complex forms the anchor for a cascade of protein-protein interactions required for exocytosis to occur.

Syntaxin and SNAP-25 bind tightly to each other and form a high-affinity binding site for synaptobrevin/VAMP. The resulting core complex has a 1:1:1 stoichiometry and is SDS resistant²⁷. Under steady-state conditions, most syntaxin, synaptobrevin and SNAP-25 in the nerve terminal are not assembled together. Synaptobrevin is largely bound to synaptophysin, an SV protein^{31–33}. At least some of the syntaxin is bound to munc18 (ref. 34) (also called n-secl³⁵ and rb-secl³⁶) over the entire plasma membrane.

Munc18 is the mammalian homologue of the proteins encoded by the *Caenorhabditis elegans unc-18* and the *Drosophila rop* genes and is distantly related to the yeast SEC1, SLY1 and SLP1 proteins^{34–36}. Mutations in the *Drosophila* and *C. elegans* munc18 homologues lead to severe synaptic defects, suggesting an essential function of munc18 in SV exocytosis^{37,38}. One of

the functions of the munc18/syntaxin and the synaptophysin/synaptobrevin interactions may be to inhibit the formation of the synaptic core complex which would otherwise assemble spontaneously and indiscriminately outside the active zone²⁷. Binding of munc18 to syntaxin inhibits its interaction with SNAP-25 (ref. 29), and the interaction of synaptophysin with synaptobrevin is incompatible with the binding of synaptobrevin to the SNAP-25/syntaxin complex³³. This suggests that synaptophysin and munc18 have to dissociate from synaptobrevin and syntaxin, respectively, before the core complex can assemble.

After SV docking, munc18 probably dissociates from syntaxin, and synaptophysin from synaptobrevin (Fig. 4a, b). This allows assembly of the synaptic core complex, which spontaneously transforms into an SDS-resistant conformation (Fig. 4c, d)²⁷. The phenotype of the munc18 mutants in *Drosophila* and *C. elegans* suggests that munc18 is important in the assembly³⁸. Munc18 may collaborate with rab3 and/or other rab proteins to ensure that the synaptic core complex is assembled only at the active zone. This hypothesis is suggested by genetic interactions between homologues for these proteins in yeast^{39,40}, and by the similarity between rab3A-deficient mice and *Drosophila*

BOX 3 Mutations and toxins that disrupt the synaptic vesicle cycle

(1) Mutations

Gene products	Species	Phenotype
ACh transporter	<i>C. elegans (unc-17)</i>	paralysed; aldicarb resistant ¹¹¹
CSP	<i>Drosophila</i>	lethal ⁸⁶
Dynamin	<i>Drosophila (shibire)</i>	lethal ⁷⁷⁻⁷⁹
Munc13	<i>C. elegans (unc-13)</i>	paralysed; aldicarb resistant ¹¹⁵
Munc18	<i>C. elegans (unc-18)</i>	paralysed; aldicarb resistant ³⁷
	<i>Drosophila (rop)</i>	lethal; general secretory defect ³⁸
	Mice	lethal*
Rab3A	Mice	synaptic depression during repetitive stimulation ²¹
Synapsin I	Mice	increased paired-pulse facilitation ¹⁰³
Synapsin II	Mice	decreased post-tetanic potentiation; synaptic depression ¹⁰⁴
Synaptotagmin	<i>C. elegans (snt-1)</i>	mildly paralysed; aldicarb resistant ¹¹⁷
	<i>Drosophila (syf)</i>	lethal; severely paralysed ^{59,60}
	Mice	severe defect in fast Ca ²⁺ -dependent SV exocytosis ⁵⁴
Syntaxin	<i>Drosophila</i>	lethal; general secretory defect ¹¹⁸

(2) Toxins

Toxin	Targets	Toxin action
Tetanus toxin	Synaptobrevins/VAMPs	Block neurotransmitter release ^{44,45}
Botulinum toxins B, D, F, G		
Botulinum toxin C1	Syntaxin	Blocks neurotransmitter release ^{44,45}
Botulinum toxins A, E	SNAP-25	Blocks neurotransmitter release ^{44,45}
α -Latrotoxin	Neurexin α ?†	Triggers Ca ²⁺ -independent SV exocytosis ⁶⁶
α -Latroinsectotoxin	Insect neurexin?	Triggers Ca ²⁺ -independent SV exocytosis ¹¹⁹

* M. Verhage and T.C.S., unpublished data.

† Neurexin α constitutes a high-affinity cell-surface receptor for α -latrotoxin which binds α -latrotoxin in a Ca²⁺-dependent manner but α -latrotoxin also stimulates release in the absence of Ca²⁺, making it unclear if neurexin α constitutes the receptor responsible for release.

overexpressing the munc18-homologue rop. Both of these exhibit a synaptic depression during repetitive stimulation which is indicative of a defect in docking and fusion of SVs^{21,41}.

Once the trimeric core complex has assembled, it serves as a receptor for SNAP and NSF (SNARE)^{26,30} (Fig. 4e, f). NSF is a *N*-ethylmaleimide-sensitive ATPase with a universal function in membrane fusion⁴². α -, β - and γ -SNAPs are soluble NSF-attachment proteins that are not related to SNAP-25 (see Box 2) (ref. 43). Synaptobrevin, syntaxin and SNAP-25 were affinity purified on immobilized α -SNAP (ref. 26), but individually bind only weakly to α -SNAP. Only their assembly into the core complex results in the formation of a high-affinity binding site for α -SNAP³⁰ which in turn creates a receptor site for NSF. As a trimer, NSF then probably multimerizes core complexes and disrupts them under ATP hydrolysis (Fig. 4g)^{28,42}. Thus an ordered sequence of protein-protein interactions leads to the assembly of a multimeric complex that is then disrupted by the enzymatic activity of NSF.

Inhibition of priming by toxins

Botulinum and tetanus toxins enter nerve terminals and irreversibly inhibit SV exocytosis, thereby incapacitating the victim. The toxins are proteases, each of which cleaves a single target at a single site. Of eight toxins, five digest synaptobrevin, two SNAP-25 and one syntaxin^{44,45}. Even although the eight different toxins are homologous to each other, their target proteins are not. Moreover, SNAP-25 and synaptobrevin are cleaved by multiple toxins at distinct sites that are not similar.

Protein cleavages by different toxins exert distinct effects on the formation of the synaptic core complex. The cleavages either sever one of the membrane anchors of the core complex, block its assembly, or inhibit its transformation into an SDS-resistant form²⁷. This suggests that the formation of the synaptic core complex is essential for SV exocytosis. The core complex bridges the space between SVs and the plasma membrane, suggesting that the interactions between syntaxin, SNAP-25 and synaptobrevin may determine the specificity of docking (the SNARE

hypothesis⁴⁶). However, nerve terminals poisoned by botulinum or tetanus toxins exhibit a normal morphology with slightly increased, not decreased, numbers of docked SVs, despite low levels of continued release^{47,48}, and the distribution of syntaxin and SNAP-25 show little specificity for the site of exocytosis, the active zone⁴⁹. Thus the primary function of the core complex seems to be SV fusion and not docking.

Several observations suggest that NSF disrupts the core complex under ATP hydrolysis before the Ca²⁺-triggered final fusion step. In adrenal chromaffin cells, Ca²⁺-dependent exocytosis does not require ATP initially, but 'priming' of continuous exocytosis does⁵⁰. ATP-independent exocytosis is not inhibited by tetanus or botulinum A toxin, but ATP-primed exocytosis is blocked⁵¹. Furthermore, the components of the core complex can only be cleaved by tetanus or botulinum toxins before assembly, but not in the complex²⁷. Thus a model emerges whereby SVs are first docked by an unidentified mechanism that generates specificity, are then primed in a partial fusion reaction that involves assembly of the synaptic core complex and its disruption by NSF, and are finally triggered to fuse by Ca²⁺ (Fig. 4).

The synaptic core complex probably consists of a stable coiled-coil structure²⁷. Why is such a tight complex first assembled and then disrupted? It is possible that the disruption of multimerized core complexes by NSF takes the fusion reaction up to the point of hemifusion, a state where one of the two leaves of the bilayer has fused. Recent experiments using mutant influenza haemagglutinin demonstrated that hemifusion states are stable⁵². This speculative hemifusion state is drawn in Fig. 4h as mediated by synaptobrevin, which has a highly amphipathic amino-terminal sequence resembling fusion peptides of viral fusion proteins. Hemifused SVs at the active zone would allow fast Ca²⁺-triggered fusion because half of the fusion reaction would already have been completed.

Synaptotagmins

SVs are docked close to Ca²⁺ channels at the active zone. This arrangement facilitates rapid exocytosis by high local concentra-

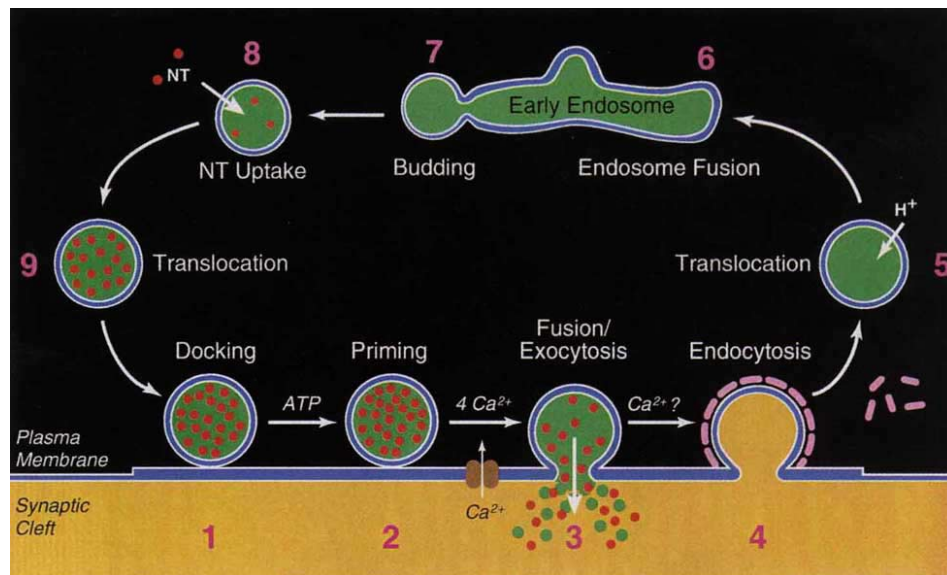


FIG. 1 The nine steps of the synaptic vesicle (SV) cycle in the presynaptic nerve terminal.

tions of Ca^{2+} during an action potential. However, signal transduction leading to exocytosis is inefficient. On average, 3 to 10 pulses of Ca^{2+} are required to trigger the exocytosis of a single SV, although 10 to 30 SVs are docked at most synapses at any given time⁵⁻⁷. The probability of exocytosis exhibits a nonlinear dependence on the Ca^{2+} concentration with an apparent cooperativity of 4 (ref. 53).

To trigger the final stage of the fusion reaction, a Ca^{2+} sensor is required at the site of exocytosis. This sensor must bind Ca^{2+} cooperatively and undergo a Ca^{2+} -dependent conformational change. If this Ca^{2+} sensor could be identified, it would give the first mechanistic clue to the regulation of the fusion reaction.

Five lines of evidence suggest that synaptotagmins function as such sensors in the final fusion step. (1) Biochemically, synaptotagmins are SV proteins that bind Ca^{2+} cooperatively and undergo a Ca^{2+} -dependent conformational change⁵⁴⁻⁵⁶. (2) In synaptotagmin-I knockout mice, Ca^{2+} -dependent neurotransmitter release is severely impaired⁵⁷. The fast, synchronous component of Ca^{2+} -triggered release is inhibited, but the slow, asynchronous component is unimpaired. (3) In the synaptotagmin-I knockouts, release can still be triggered normally by agents that act by Ca^{2+} -independent mechanisms, such as hypertonic sucrose or α -latrotoxin⁵⁷. However, in nerve terminals poisoned by tetanus and botulinum A toxins, the response to

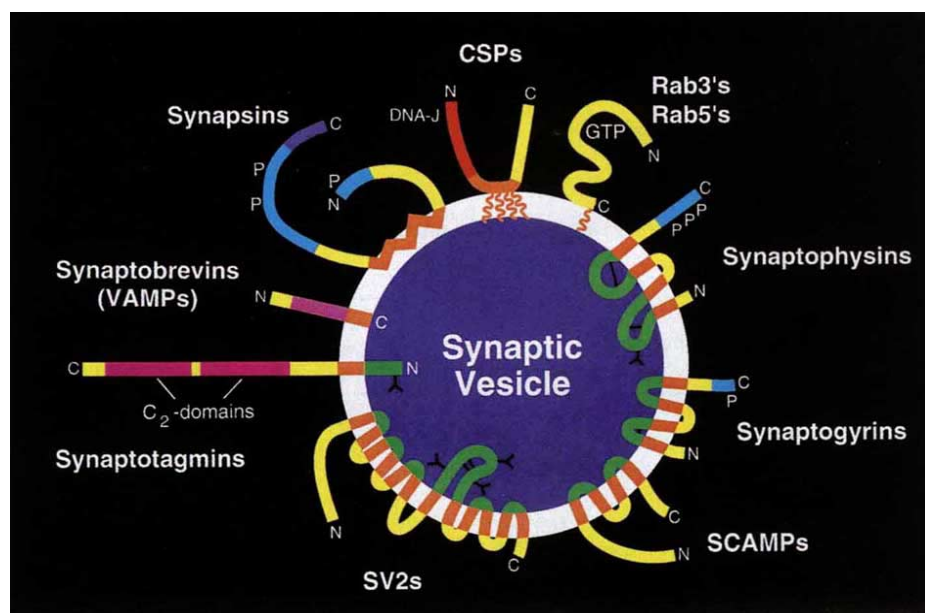


FIG. 2 Structures of the major proteins of SVs with putative functions in SV trafficking. Proteins are depicted schematically in scale. Only one isoform for each protein family is represented. Isoprenyl groups that attach rab proteins and palmitoyl chains that bind cysteine string

proteins (CSPs) to SVs are depicted as thin orange lines. The N termini of proteins are marked by N, the C termini by C. P indicates positions of phosphorylation sites. See Box 2 for a glossary of the different proteins.

FIG. 3 The rab3 cycle. GTP-bound rab3A or rab3C (rab3A/C) associates with SVs (top). SVs then bind rabphilin-3A (left). When GTP is hydrolysed to GDP, rabphilin-3A dissociates from rab3 (bottom; it is uncertain if rabphilin-3A physically dissociates from SVs). GTP hydrolysis is shown here to be associated with docking, but rab3A function in SV traffic could also occur at a later stage of the cycle. After GTP hydrolysis, GDI binds to GDP-rab3 and removes it from the membrane (bottom middle). The Rab3-GDP/GDI complex reassociates with SVs at an unknown stage of the pathway (top), resulting in GDP/GTP exchange and dissociation of GDI. Thus the rab3 cycle contains four nested cycles that are identified by different colours: the SV cycle (blue), the rab3 cycle (red), the rabphilin-3A cycle (gold) and the GDI cycle (pink).

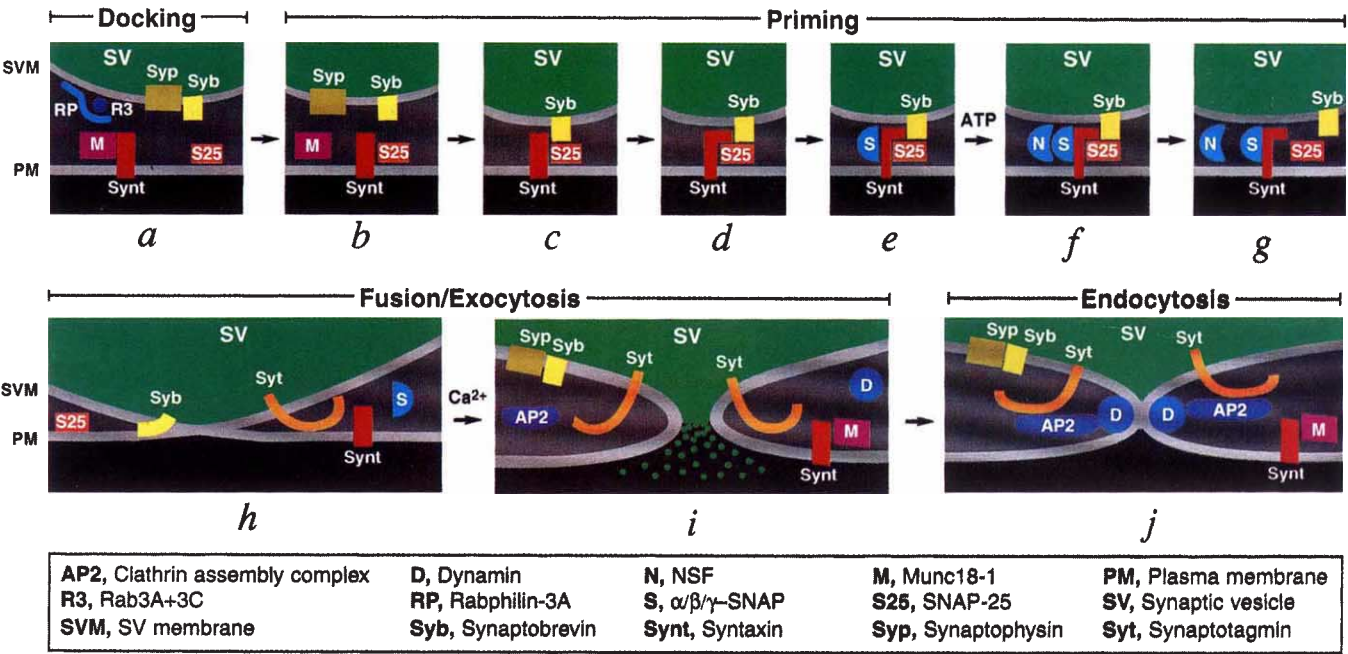
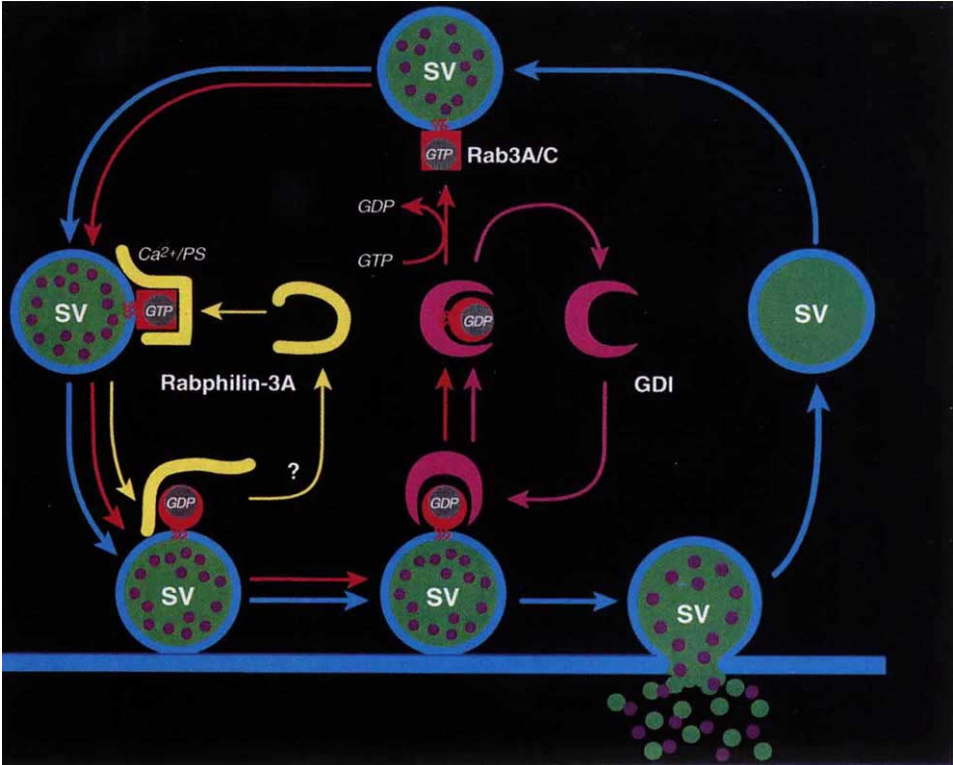


FIG. 4 Protein-protein interaction cascades that mediate SV exo- and endocytosis. Top panels (A–G) show a model for the assembly and disruption of the synaptic core complex. The bottom panels (H–J) exhibit a speculative proposal for a string of reactions mediating Ca^{2+} -triggered exocytosis and rapid endocytosis. Top panels. When docking occurs, syntaxin is complexed to munc18, and synaptobrevin to synaptophysin (A). These complexes dissociate during or after docking by an unknown mechanism (B). SNAP-25 then forms a complex with syntaxin, thereby creating a high affinity binding site for synaptobrevin which results in the synaptic core complex (C) which becomes SDS-resistant (D). The synaptic core complex serves as a receptor for SNAPS (E), which in turn binds NSF (F). NSF as a trimer crosslinks multiple core complexes into

a network and disrupts the core complex with ATP hydrolysis (G). Botulinum and tetanus toxins would inhibit C, and N-ethylmaleimide stage G. Bottom panels. ATP hydrolysis by NSF disrupts the core complex and leads to hemifusion, possibly by means of the amphipathic N terminus of synaptobrevin (H). Synaptotagmin then acts as a Ca^{2+} sensor in completing the fusion reaction by a Ca^{2+} -triggered interaction with syntaxin (J). Immediately after fusion, synaptotagmin acts as a nucleus for clathrin coat assembly by serving as an AP2 receptor (J). Clathrin and/or dynamin binding to AP2 then drive endocytosis. Hypertonic sucrose and α -latrotoxin probably activate Ca^{2+} -independent fusion after stage C but before the action of synaptotagmin (stage H–J).

hypertonic sucrose is blocked⁴⁷. This suggests that synaptotagmin I acts after the core complex that is inhibited by these toxins. (4) In squid nerve terminals injected with synaptotagmin peptides, release is inhibited and SVs accumulate at the active zone⁵⁸. This presumably results from competition by the peptides for synaptotagmin effectors. (5) Synaptotagmin mutants in *Drosophila* show a severe but incomplete block of neurotransmission with an altered Ca^{2+} dependence in some mutants^{59,60}. Similar changes are also observed with manipulations of synaptic transmission that are unrelated to the Ca^{2+} sensor, making an interpretation of such changes difficult.

How do synaptotagmins trigger fast release? No changes in the frequency of spontaneous SV exocytosis ('minis') were observed in synaptotagmin-I knockouts, suggesting that synaptotagmin is not simply arresting membrane fusion but plays an active role⁵⁸. Synaptotagmin I binds phospholipids as a function of Ca^{2+} with half-maximal binding at 3–6 μM free Ca^{2+} , an affinity that is higher than the low apparent Ca^{2+} affinity of the Ca^{2+} sensor in SV exocytosis (>100 μM)^{54–56,61}. Syntaxin, a component of the synaptic core complex involved in priming, directly interacts with synaptotagmin I (refs 62, 63). Recent studies showed that syntaxin binding to synaptotagmin I is regulated by Ca^{2+} but requires more than 200 μM Ca^{2+} for half-maximal binding⁶⁴. This approximates the Ca^{2+} requirement of SV exocytosis and suggests a mechanism whereby Ca^{2+} triggers SV exocytosis by regulating synaptotagmin-I interaction with syntaxin or other target proteins. The Ca^{2+} -dependent phospholipid binding by synaptotagmin may remodel membrane phospholipids during exocytosis and transform a hemifused into a fused membrane. However, Ca^{2+} -triggered fusion is probably a complex reaction involving multiple proteins and not only the interaction of synaptotagmins with syntaxins. In addition to phospholipids and syntaxins, synaptotagmin I binds to the cytoplasmic domains of neurexins, which are polymorphic cell-surface proteins that are expressed exclusively in neurons (Box 3) (refs 65, 66). The neurexins and other, as yet unidentified, interacting molecules may contribute to shaping the fusion reaction.

Surprisingly, brain expresses at least seven other synaptotagmins in addition to synaptotagmin I, four of which are also present in non-neural cells^{64,67–70}. The different synaptotagmins are differentially expressed in neurons with partly overlapping patterns, suggesting that different synaptotagmins may have distinct functions. Such a functional heterogeneity is supported by the differential Ca^{2+} -binding properties of synaptotagmins⁶⁴. Some synaptotagmins appear to be independent of Ca^{2+} (synaptotagmins IV, VI and VIII), others exhibit a similar high Ca^{2+} affinity for syntaxin and for phospholipid binding (synaptotagmins III and VII), and a third class binds syntaxin only at high Ca^{2+} concentrations but phospholipids at low Ca^{2+} concentrations (synaptotagmins I and II). Interestingly, all three classes appear to be coexpressed in neurons, suggesting functional differentiation. This is supported by the inability of synaptotagmin III, which is coexpressed with synaptotagmin I in the hippocampal neurons analysed in the synaptotagmin I knockout, to substitute for synaptotagmin I. The first two classes of synaptotagmins are also present in non-neural cells, whereas the last class, exemplified by synaptotagmin I, is specialized for fast Ca^{2+} -dependent exocytosis. Together these findings suggest that synaptotagmins may have a general function in cellular membrane fusion in collaboration with non-neural isoforms of syntaxin, with different synaptotagmins being specialized for distinct reactions.

Endocytosis of synaptic vesicles

Empty SV membranes are taken up after exocytosis in 1 to 5 s (ref. 71), much faster than most endocytic events. Similar endocytosis is also observed in neuroendocrine cells⁷². Two mechanisms have been proposed: endocytosis via coated pits akin to receptor-mediated endocytosis⁷³, or coupled exo- and

endocytosis ('kiss-and-run')⁷⁴. Rapid endocytosis is faster than receptor-mediated endocytosis in fibroblasts and is not inhibited by decreases in the pH or the K^{+} concentration which disrupt receptor-mediated endocytosis⁷². These findings suggest that receptor-mediated endocytosis in fibroblasts and rapid endocytosis of SVs are different processes. Nevertheless, three observations support a general role for clathrin-coated vesicles in SV endocytosis. (1) Coated vesicles from brain are largely coated SVs^{75,76}. Moreover, coated vesicles are abundant in nerve terminals and increase more than tenfold after stimulation⁸. (2) Endocytosis of SVs and other membranes is disrupted in the paralytic *Drosophila* mutant *shibire*⁷⁷. Paralysis correlates with a loss of SVs, an increase in the nerve terminal area, and the appearance on the plasma membrane of coated pits that do not bud. Cloning of the *shibire* gene demonstrated that it encodes a *Drosophila* homologue of dynamin^{78,79}, a protein that functions in clathrin-mediated endocytosis. (3) Dynamin I is a major phosphoprotein of the synapse that is quantitatively dephosphorylated in a Ca^{2+} -dependent manner upon depolarization⁸⁰. Dephosphorylation inhibits the GTPase activity of dynamin I, suggesting that dynamin I is regulated in conjunction with SV endocytosis. Furthermore, incubation of permeabilized synaptosomes with a non-hydrolysable analogue of GTP (GTP- γS) results in the assembly of dynamin coats on invaginated empty SVs on the plasma membrane⁸¹.

The most plausible hypothesis to resolve the different findings is that nerve terminals utilize a novel type of clathrin-mediated endocytosis that has kinetics and biochemical characteristics distinct from receptor-mediated endocytosis in fibroblasts. This hypothesis is supported by the presence in nerve terminals of special clathrin-associated proteins, such as AP180 and auxilin⁸², which might modify the pH and K^{+} sensitivity and the kinetics of clathrin assembly. SV exocytosis is stimulated by α -latrotoxin, a component of black-widow spider venom. In the absence of Ca^{2+} , α -latrotoxin still stimulates exocytosis but subsequent endocytosis is inhibited⁸³. A Ca^{2+} -dependent step in endocytosis could consist of the Ca^{2+} -dependent dephosphorylation of dynamin I (ref. 80).

Fast clathrin-mediated endocytosis would require clathrin assembly. Assembly of clathrin coats is thought to be initiated by binding of the AP2 adaptor protein complex to membrane receptors. A search for such a receptor on SVs revealed that AP2 binds to synaptotagmin with high affinity⁸⁴. The AP2 binding suggests a role for synaptotagmin in the endocytotic reaction that immediately follows exocytosis. Because SVs only have a limited number of proteins to guide them through a complex life cycle, it would be economical for a single protein to function both in the last step of exocytosis and the first step of endocytosis, and to couple exo- and endocytosis to each other. The ubiquitously distributed synaptotagmins may serve as general AP2 receptors in all cells⁶⁴. AP2 also binds a dynamin I, AP180 and amphiphysin, suggesting that AP2 may be the linchpin of a macromolecular complex that attaches to synaptotagmin and mediates fast clathrin-dependent endocytosis⁸⁵.

Cysteine string proteins (CSPs) are SV proteins that are attached to SVs by a string of more than ten palmitoylated cysteines. Mutations in the *Drosophila* homologue of CSP lead to a decrease in evoked neurotransmitter release⁸⁶. CSP contains a DNA-J homology domain that in other proteins acts cooperatively with heat-shock protein 70 (Hsp70) as a molecular chaperone. The only known function for an Hsp70 in the SV pathway is the uncoating of clathrin-coated vesicles, which is thought to be mediated by Hsp70 (ref. 87). CSP may facilitate rapid decoating by cooperating with Hsp70. Such a function would be consistent with a decrease in SVs in the nerve terminal of CSP mutants.

Synaptic plasticity

Alterations in synaptic strength, referred to as synaptic plasticity, have presynaptic and postsynaptic origins. Elegant *in vivo*

experiments revealed an important role for protein kinases in initiating synaptic plasticity⁸⁸⁻⁹¹. However, long-term changes are unlikely to be maintained by covalent modifications of a few proteins; they are more likely to involve restructuring of synapses and changes in gene expression. Although such changes require nuclear signals, they nevertheless remain restricted to a few selected synapses of a given neuron.

On the presynaptic side, synaptic plasticity is characterized by changes in neurotransmitter release. These could be caused by changes in Ca^{2+} influx during an action potential, or by direct effects on the fusion machinery which modulate neurotransmitter release probability. Several protein kinase substrates have been characterized that may function in synaptic plasticity: most prominent among these are the synapsins, a class of abundant membrane proteins of SVs (Fig. 2)⁹².

Synapsins Ia, Ib, IIa and IIb are the alternatively spliced products of two genes, and together account for approximately 9% of the SV protein⁹³. Synapsins are phosphorylated at their N terminus by Ca^{2+} , calmodulin-dependent protein kinase I (CaM KI) and cAMP-dependent protein kinase. In addition, synapsin I but not II contains C-terminal phosphorylation sites for CaM KII. Synapsin I binds with high affinity to several proteins *in vitro*, including neurofilaments, actin filaments, spectrin, annexin VI, grb2, calmodulin, CaM KII and tubulin⁹⁴⁻¹⁰⁰. Synapsin interactions with actin filaments and neurofilaments are regulated by the C-terminal CaM KII phosphorylation, and annexin VI binding by Ca^{2+} . In squid neurons, injection of synapsin I that was not phosphorylated at the C terminus resulted in inhibition of neurotransmitter release, whereas injection of phosphorylated synapsin I had no effect¹⁰¹. This suggests that an excess of synapsin I 'freezes' the nerve terminal in a phosphorylation-dependent manner. Conversely, injections of synapsin II into *Xenopus* oocytes resulted in strengthening of the synapses formed by their developing neurons¹⁰². Together these data indicate positive and negative roles for synapsins in regulating SV traffic. This was confirmed in studies of gene-targeted mice lacking one or both synapsins^{103,104}.

Mice lacking synapsins are viable and fertile, and have a normal synaptic architecture. However, they are prone to seizures and are unable to regulate synaptic transmission properly. In mice lacking synapsin I, a selective increase in paired pulse facilitation is observed, but not in other forms of synaptic plasticity. Paired pulse facilitation consists of the potentiation of release during the second of two closely spaced action potentials and occurs on a millisecond timescale. Mice lacking synapsin II had normal paired pulse facilitation but exhibited a major decrease in a different form of short-term synaptic plasticity, post-tetanic potentiation¹⁰³. This is the potentiation of neurotransmission that is induced by tetanic stimulation, and lasts for several seconds. In addition, repetitive stimulation of synapses at physiological frequencies caused massive synaptic depression. This suggests that the SV cycle is unable to accelerate appropriately upon repetitive stimulation in the synapsin-II knockout¹⁰⁴. The synapsin-I knockout did not exhibit decreased post-tetanic potentiation or synaptic depression upon repetitive stimulation, but aggravated this phenotype in the double knockout.

Thus synapsins are not essential for synaptic development or SV traffic but are required for synaptic regulation. The most plausible model to explain the results of the synapsin-II knockout and injections is that synapsin II is a positive regulator of neurotransmitter release which is activated during accelerated SV traffic by repetitive firing. Synaptic depression during repetitive stimulation was even more severe in the double knockout when expression of both synapsins was deleted, suggesting that synapsin I, like synapsin II, also functions as a positive regulator. Repetitive stimulation increases nerve terminal Ca^{2+} levels and activates CaM kinases. That synaptic depression is observed in the synapsin knockouts under these conditions raises the possibility that positive regulation of release by synapsins involves

the N-terminal phosphorylation sites for CaM KI in synapsins I and II.

Why then does the synapsin I knockout not show the same synaptic depression that it aggravates in the synapsin II knockout, and why is paired pulse facilitation not affected in the double knockout? There is currently no conclusive answer to this puzzle. In a knockout, the phenotype observed is the sum of all biological activities for which a given protein is essential but does not report redundant functions. The increase in paired pulse facilitation in the synapsin-I knockout suggests that synapsin I normally inhibits neurotransmitter release on a millisecond timescale immediately after a Ca^{2+} signal, possibly by means of the C-terminal phosphorylation by CaM KII. This effect may not be apparent in the double knockout because the depression of release may already occur in the second stimulus. Conversely, the synapsin-I knockout may not exhibit synaptic depression during repetitive stimulation because the inhibitory effect of synapsin I that documents itself in increased paired pulse facilitation may balance its positive effect.

The essential function of the synapsins is restricted to regulating SV traffic. Thus an understanding of their mechanism of action would give insights into synaptic regulatory mechanisms. In the synapsin double knockout, no major redistribution of SVs was observed¹⁰⁴. The regulatory changes of release in the synapsin knockouts set in very rapidly during repetitive stimulation, long before docked SVs are exhausted. These observations suggest that the action of synapsins occurs after docking of SVs. Interestingly, in the synapsin knockouts the impaired regulation of SV traffic leads to a destabilization of SVs and a decrease in the levels of some but not all SV proteins. Intrinsic membrane proteins are decreased, but rab3A and rabphilin-3a (which dissociate from SVs during or after docking and/or exocytosis; see Fig. 3) are not. Rab5, which has a probable function in the endosome, is even increased¹⁰⁴. This pattern also supports the idea that the impairment of SV traffic in the knockouts is localized to a step distal to docking but does not exclude a role for the cytoskeleton in this regulation.

Perspectives

Synaptic connections between neurons are specific and not formed at random. They consist of precisely aligned pre- and postsynaptic specializations. There must be a recognition process that establishes the first contact, and a reinforcement process that maintains it. Different synapses formed by a neuron have distinct release probabilities regulated by the target. Thus synapses not only mediate fast, point-to-point transmission of an intercellular signal in a single direction but also represent bidirectional junctions that are plastic. Structurally they resemble tight junctions in which the cytoplasmic domains of intercellular adhesion molecules are nucleating submembrane coats. The cell-adhesion mechanisms involved are asymmetric and heterotypic. They must at least partly explain the specificity of synapse formation that discriminates among millions of neurons. A recently identified family of polymorphic cell-surface proteins comprising more than 1000 isoforms, the neuroligins⁶⁶, may represent the first candidates for cell-surface proteins that could confer such specificity.

Insight into the SV pathway is important for our understanding of fundamental mechanisms of membrane transport and the design and properties of neural networks. At this point, the molecular description of the SV pathway has reached the sophistication of early cartography: we can delineate contours of continents but the interiors are largely blank. Proteins such as synaptotagmin or dynamin have been identified that perform functions localized to distinct steps in the pathway. However, it is unknown how these proteins act. Moreover, the functions of several important synaptic proteins, for example that of synaptophysin or SV2s, remain to be explored, whereas no candidate proteins have been proposed for many steps in the pathway (Fig.

1 and Box 2). Clearly we are only beginning to understand the SV cycle, its regulation and its topologic specificity. Several major problems must be addressed, including: (1) how specificity is achieved in the docking of SVs; (2) how membranes fuse, and how different steps of the fusion reaction (priming, Ca^{2+} -triggered fusion) are regulated; (3) what the composition of the active zone is, and how it is assembled in complementarity with the postsynaptic specializations; and (4) what proteins function

during SV recycling, and how many proteins are required for each distinct step in the SV cycle.

Decisive solutions should emerge from biochemical, morphological and electrophysiological analyses of the appropriate genetic models in mice, *Drosophila* and *C. elegans*. □

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Polarization of both major body axes in *Drosophila* by *gurken*–*torpedo* signalling

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Anterior–posterior polarity in *Drosophila* arises from the movement of the oocyte to the posterior of the egg chamber, and the subsequent acquisition of posterior fate by the adjacent somatic follicle cells. We demonstrate that *gurken* is necessary in the oocyte and *torpedo*/DER in the follicle cells for the induction of posterior fate. As the *gurken*–*torpedo*/DER pathway also establishes dorsoventral polarity later in oogenesis, *Drosophila* uses the same germline to soma signalling pathway to determine both embryonic axes.

THE polarities of both the anterior–posterior (AP) and dorsal–ventral (DV) axes of *Drosophila* are determined during oogenesis as a result of inductive interactions between the germline cells of the egg chamber, the oocyte and the nurse cells, and the surrounding layer of somatic follicle cells (for a review of oogenesis see ref. 1). The first visible sign of AP asymmetry is the movement of the oocyte to the posterior of the nurse cells. Once the oocyte has reached this position, it induces the adjacent polar follicle cells to adopt a posterior fate rather than the default anterior fate². Later in oogenesis, the posterior follicle cells signal back to the oocyte to polarize its microtubule cytoskeleton, so the ‘minus’ ends lie at the anterior pole and the ‘plus’ ends at the posterior, thereby directing the microtubule-dependent localization of *bicoid* (*bcd*) and *oskar* (*osk*) messenger RNAs to opposite poles of the oocyte^{2–10}. Because *bcd* mRNA encodes the anterior determinant, and the localization of *osk* mRNA defines the site of formation of pole plasm, which contains the posterior and germline determinants^{11,12}, the specification of the posterior follicle cells defines the AP polarity of the resulting embryo. Thus AP axis formation requires two sequential inductions, first from the germ line to the somatic follicle cells, and then from the follicle cells back to the oocyte. However, the signalling molecules responsible for these two inductions have not yet been identified.

The formation of the DV axis of the embryo involves a similar series of inductions between the germ line and the follicle cells. During stages 8 and 9 of oogenesis, the oocyte induces the follicle cells on one side of the egg chamber to adopt a dorsal fate¹³. This induction requires the activity of *gurken* (*grk*) in the germ line and *torpedo*/DER (*top*/DER) in the follicle cells¹³. As *grk* encodes a transforming growth factor (TGF)- α -like protein that contains an epidermal growth factor (EGF) repeat¹⁴, while *Top*/DER is the *Drosophila* homologue of the EGF receptor^{15,16}, it has been proposed that the Gurken protein binds to the *Top*/DER protein in the adjacent follicle cells to activate a receptor tyrosine kinase signal transduction pathway that results in the determination of dorsal fate^{14,17,18}. The resulting polarization of the follicle cell layer determines the embryonic DV axis, as the ventral follicle cells will subsequently signal back to the germ line to define the high point of the dorsal nuclear gradient on the ventral side of the embryo¹⁹.

Although embryos laid by *grk* mutant females have been reported not to have AP defects²⁰, the initial description of the mutant phenotype suggested that *grk* might also be involved in the generation of AP polarity in the follicle cell layer¹³. In wild-type egg chambers, the anterior follicle cells produce a distinct structure called the micropyle at the anterior end of the egg, and the posterior follicle cells secrete the acropyle. However, the eggs laid by *grk* mutant mothers often possess a second micropyle at the posterior pole¹³. Here we show that this phenotype is a result

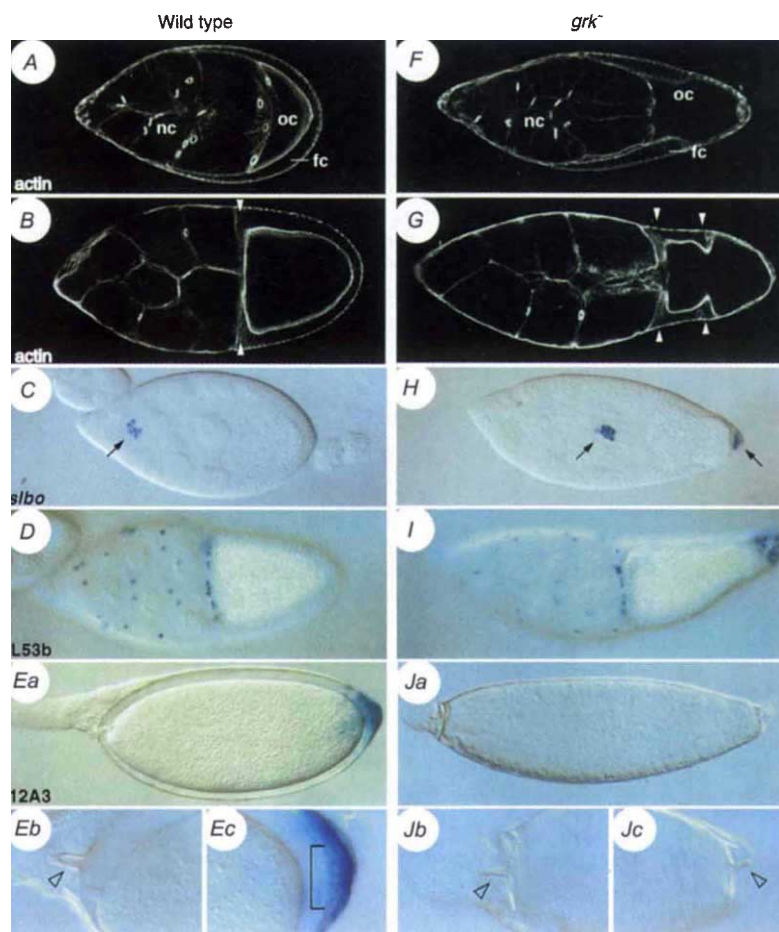
of a duplication of anterior follicle cells at the posterior pole of the egg chamber. Because the AP phenotype of *grk* mutants is dependent on the germ line, we propose that *grk* encodes the inductive signal produced by the oocyte to determine posterior follicle cell fate. As a consequence of the lack of posterior follicle cells, the oocyte microtubule cytoskeleton develops a symmetric organization that results in the localization of *bcd* mRNA to both poles of the oocyte, and of *osk* mRNA to the centre. Because reduction of *top*/DER activity in the follicle cells gives rise to the same phenotype, we conclude that Gurken signals to *Top*/DER to establish the polarity of the AP axis. The polarization of the oocyte cytoskeleton is required for the subsequent microtubule-dependent movement of the germinal vesicle to the dorsal anterior margin of the oocyte. As the position of the germinal vesicle determines the site of localization of *grk* mRNA, and hence where the dorsal follicle cells are induced, the formation of the DV axis depends on the earlier polarization of the AP axis.

AP polarity requires *grk*

Several subpopulations of follicle cells can be distinguished along the AP axis of wild-type egg chambers¹ (Fig. 1A–E). At stage 9 of oogenesis, a cluster of follicle cells at the anterior tip of the egg chamber, the border cells, start to express the *slow border cells* (*slbo*) gene and delaminate from the follicle cell layer to migrate between the nurse cells to the anterior margin of the oocyte²¹ (Fig. 1C). The adjacent anterior follicle cells become stretched to cover the nurse cells, as the rest of the follicle cells migrate posteriorly to form a columnar epithelium around the oocyte. Later, the anteriormost columnar follicle cells migrate centripetally between the nurse cells and the oocyte (Fig. 1A, B). These three anterior populations of follicle cells, the border cells, the stretched follicle cells and the centripetal cells, all express the L53b β -gal enhancer trap line²² (Fig. 1D). At stage 13 of oogenesis, the posterior follicle cells express the 12A3 β -gal fusion construct²³ (Fig. 1Ea, Ec).

In *grk* mutants, all three anterior follicle cell types are duplicated at the posterior end of the egg chamber. A small cluster of *slbo*-expressing cells form at the posterior pole, and a larger group of posterior cells activate the L53b enhancer trap (Fig. 1H, I). Furthermore, these ‘posterior’ cells undergo the same morphological movements as the anterior follicle cells: the *slbo*-expressing cells lose their epithelial organization to form a ball of cells, the adjacent cells become stretched, and the neighbouring columnar cells migrate centripetally, sometimes bisecting the oocyte (Fig. 1F, G). Thus the border cells, stretched follicle cells and the centripetal cells autonomously perform their normal developmental programmes when they are duplicated in reverse order at the posterior of *grk*[−] egg chambers, even though they are no longer in contact with the nurse cells. The duplication of

FIG. 1 The induction of posterior follicle cell fate requires *grk*. **A, B, F, G**, Rhodamine-labelled phalloidin staining of actin filaments. **C–E, H–J**, X-gal-stained egg chambers. An egg chamber consists of a layer of somatic follicle cells surrounding 15 nurse cells and a posterior oocyte which are interconnected by actin-rich ring canals¹. At stage 9, most of the follicle cells adopt a columnar shape and migrate posteriorly to envelop the oocyte (**A**). By stage 10, the anteriormost columnar follicle cells migrate centripetally between the nurse cells and the oocyte (**B**). Anterior to these lie the border cells, which express an enhancer trap in the *slbo* gene at stage 9 (**C**), and the adjacent stretched follicle cells. The enhancer trap line L53b expresses in the border cells, the stretched follicle cells and centripetal follicle cells at stage 10 (ref. 22) (**D**). At stage 14, the border cells and centripetal follicle cells make the micropyle (**Ea**; magnified in **Eb**), while the posterior follicle cells, which express the β -gal construct 12A3 (ref. 23) (**Ea**), secrete the aeropyle (**Ec**). **F–J**, All of the *grk*^{2E12}/*grk*²⁸⁶ egg chambers show a duplication of all three anterior follicle cell types at the posterior pole. The follicle cells at the posterior migrate anteriorly at stage 9 (**F**), some stretch over the oocyte and others migrate centripetally at stage 10 (**G**). There is expression of *slbo* at the posterior at stage 9 (**H**) and L53b at stage 10 (**I**), but 12A3 expression is absent (**J**). These ectopic anterior follicle cells secrete a second micropyle at the posterior pole of the egg, in place of the aeropyle (**Ja, b, c**). Abbreviations and symbols: fc, follicle cells; nc, nurse cells; oc, oocyte; arrowheads, centripetal follicle cells; arrows, border cells; open arrowheads, micropyle; bracket, aeropyle. In all figures, anterior is to the left and dorsal up.



anterior fates at the expense of posterior follicle cell fates is also apparent later in oogenesis. The follicle cells at the posterior pole do not express the posterior marker 12A3 and produce a second micropyle instead of the aeropyle (Fig. 1J). The changes in follicle cell fates in mutant egg chambers indicate that *grk* is required for the induction of posterior follicle cell fate by the oocyte.

To distinguish whether *grk* activity is required for the production of the inductive signal by the oocyte or for the reception of this signal in the somatic follicle cells, pole cells were transplanted to generate chimaeric egg chambers in which the oocyte and nurse cells are mutant for *grk*, but the follicle cells are *grk*⁺. Removal of *grk* in the germline cells induces ectopic expression of the anterior follicle cell marker L53b in the wild-type follicle cells at the posterior of the egg chamber (Fig. 2). Thus *grk* is required in the germ line for the induction of posterior follicle cell fate, strongly suggesting that it encodes the posterior inducer. Because *grk* mRNA is localized to the posterior margin of the oocyte during the early stages of oogenesis¹⁴, it is likely that the export of Gurken is polarized towards the responding polar follicle cells.

AP polarity in the oocyte

Later in oogenesis, the posterior follicle cells signal back to the germ line to determine AP polarity within the oocyte. If this signalling fails to occur, either as a result of loss of *Notch* or *Delta* activity in the follicle cell layer, or the absence of protein kinase A in the germ line, the oocyte develops a duplicated AP axis^{3,4}. The *grk*[−] egg chambers, which lack posterior follicle cells, also show a mirror-image duplication of the AP axis of the oocyte. Both *bcd* and *K10* mRNAs²⁴, which normally localize to just the anterior pole of the oocyte, become localized to both poles, and *osk* mRNA and Staufen protein²⁵ localize to the centre rather than the posterior (Fig. 3 and data not shown).

Because the localization of *bcd* and *osk* mRNAs is microtubule dependent^{6,8}, their altered distributions in *grk*[−] egg chambers are likely to reflect a change in the organization of the oocyte microtubule cytoskeleton. To test this possibility, we used a kinesin- β -galactosidase fusion protein as a marker of microtubule polarity²⁶. This chimaeric protein contains the motor domain of the plus-end-directed microtubule motor kinesin, and accumulates at the plus ends of the microtubules at the posterior of wild-type stage 9 oocytes⁶. In *grk*[−] egg chambers, the fusion protein localizes to the centre of the oocyte, in the same position as *osk* mRNA (Fig. 3). This indicates that the oocyte microtubule network has a symmetric organization, with the minus ends at both poles and the plus ends in the centre. Thus the induction of the posterior follicle cells by *grk* is required for the polarization of the oocyte cytoskeleton, and hence the generation of the AP axis of the embryo. Because *bcd* and *osk* mRNA localization begins at stage 7, Gurken must induce posterior fate before this stage.

AP polarity requires *top/DER*

During early oogenesis, *top/DER* mRNA is expressed throughout the follicle cell layer²⁷. We therefore investigated whether *top/DER* is required in these cells for the reception of the Gurken signal, as it is in the dorsal follicle cells later in oogenesis. Because *top/DER* is required at several stages of development²⁸, it is only possible to examine the maternal phenotype of hypomorphic mutant combinations that survive to adulthood. Nevertheless, a large proportion of such mutant egg chambers develop an AP phenotype very similar to that produced by *grk* mutations. The follicle cells at the posterior of the egg chamber behave abnormally and express anterior markers such as L53b, and the polarity of the oocyte itself is also affected: *bcd* mRNA localizes to both poles, and *osk* mRNA localizes to the centre (Fig. 4A). Because *top/DER* is not required in the

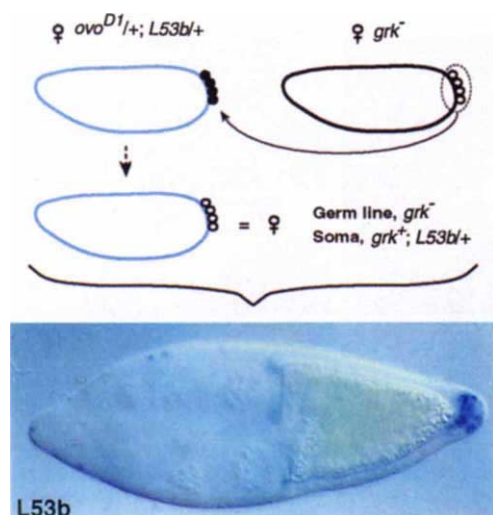


FIG. 2 The determination of posterior follicle cell fate requires *grk* in the germ line. In chimaeric egg chambers in which the germ line is mutant for *grk* and the follicle cells are wild type, the anterior marker L53b is expressed at the posterior of the follicle cell layer. These follicle cells also undergo the same morphological changes as the anterior follicle cells and produce a second micropyle.

METHODS. Males of the genotype *ovo*^{D1}/Y were crossed to females carrying the L53b enhancer trap line to generate host embryos of the genotype *ovo*^{D1}/+; *L53b*/+. Eggs laid by *grk*²⁸⁶/CyO flies (a putative null allele of *grk* (ref. 14)) were used as donor embryos. When female pole cells are transplanted into female embryos, one quarter of the chimaeric egg chambers have a germ line which is mutant for *grk*, surrounded by wild-type follicle cells which carry the L53b enhancer trap line.

germ line^{13,29}, this phenotype is presumably a consequence of the lack of gene activity in the follicle cell layer, strongly suggesting that Top/DER is the receptor for Gurken in the posterior follicle cells.

When Top/DER protein is activated at other stages of *Drosophila* development, it initiates a typical receptor tyrosine kinase signal transduction pathway that leads to the activation of the mitogen-activated protein kinase (MAPK), encoded by *rolled* (*rl*)^{30,31}. We therefore examined whether the removal of one copy of *rl* would enhance the phenotype of a weak *top/DER* mutant combination, *top*^{CA}/*top*^{QY1}, in which *osk* mRNA always localizes to the posterior of the oocyte. In 100% of *rl*^{10A}

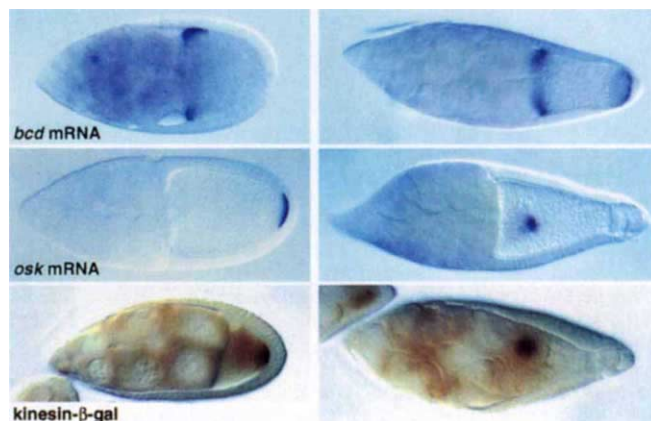


FIG. 3 The polarization of the AP axis of the oocyte requires *grk*. In wild-type egg chambers, *bcd* mRNA localizes to the anterior margin of the oocyte. In 67% of *grk*⁻ egg chambers, *bcd* mRNA localizes to both poles of the oocyte. In wild-type egg chambers, *bcd* mRNA localizes to the anterior margin of the oocyte, and *osk* mRNA to the posterior pole. In 67% of *grk*⁻ egg chambers, *bcd* mRNA localizes to both poles of the oocyte, and *osk* mRNA localizes to the centre of the ooplasm in 87% of the mutant oocytes. *osk* mRNA normally accumulates at the posterior of the oocyte, and 87% of *grk*⁻ oocytes show *osk* mRNA localized to the centre of the ooplasm. The chimaeric protein kinesin-β-gal⁶ concentrates at the posterior of stage 9 wild-type oocytes. In *grk*⁻ oocytes, the kinesin-β-gal protein accumulates in the middle.

METHODS. *In situ* hybridizations and actin staining (confocal microscopy was used to collect the images from different focal planes) as described². Staining using β-galactosidase was as described in ref. 21, and immunohistochemistry in ref. 25. We used the transgenic line KZ503 (ref. 6) to express the kinesin-β-gal protein in the oocyte. Although all the allelic combinations tested gave the *grk*⁻ phenotype (*grk*^{WG}/*grk*^{WG}, *grk*^{HK}/*grk*^{HK}, *grk*^{HK}/*grk*^{WG}, *grk*²⁸⁶/*grk*²⁸⁶, *grk*^{2E12}/*grk*²⁸⁶, *grk*²⁸⁶/*grk*^{HK} and *grk*^{2E12}/*grk*^{HK} (refs 13, 14)), all the results shown correspond to *grk*^{2E12}/*grk*²⁸⁶ egg chambers.

top^{CA}/*top*^{QY1} egg chambers, *osk* mRNA accumulates in the centre of the oocyte. This strong dominant enhancement of the *top/DER* phenotype by *rolled* suggests that the MAPK pathway is involved in the induction of posterior follicle cell fate.

AP polarity requires *cornichon*

The discovery that *grk* and *top/DER* are required for the induction of posterior follicle cell fate raised the possibility that other

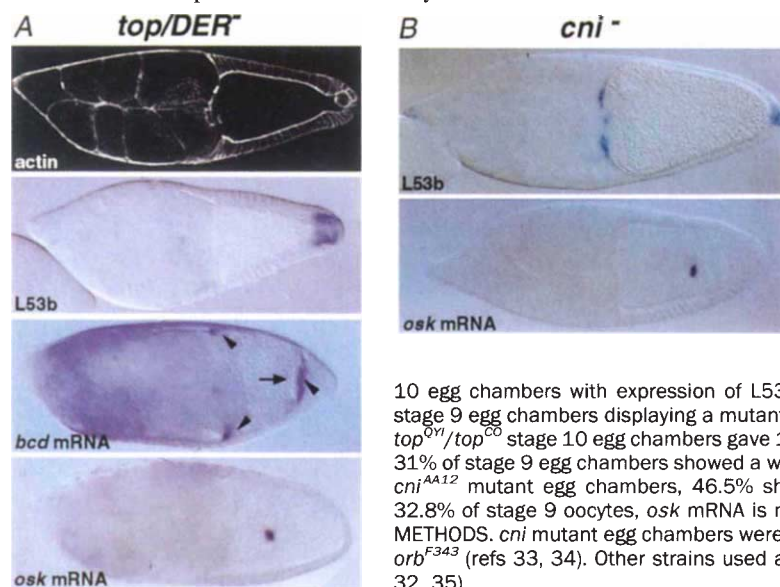


FIG. 4 Role of *top/DER* and *cni* in the establishment of AP polarity. A, The phenotype of *top*^{QY1}/*top*^{CO} egg chambers resembles that of *grk*⁻: the posterior follicle cells show an abnormal shape and behaviour and express L53b; *bcd* mRNA accumulates at both poles of the oocyte (arrowheads) and *osk* mRNA localizes to the centre of the oocyte. The arrow points to the germinal vesicle at the posterior of the oocyte. In addition to the expression of the L53b β-gal line, we have also scored *osk* mRNA localization as a measure of the strength of the phenotype and established two categories: weak cases show an incomplete and diffuse posterior localization of *osk* mRNA; in strong cases *osk* mRNA accumulates in the centre of the oocyte. The weak combination *top*^{QY1}/*top*^{QY1} gave rise to 54% of stage

10 egg chambers with expression of L53b at the posterior (3.3 stained cells on average), and 74% of stage 9 egg chambers displaying a mutant *osk* mRNA localization (60% weak phenotype and 14% strong). *top*^{QY1}/*top*^{CO} stage 10 egg chambers gave 100% expression of L53b at the posterior (22.5 cells on average); 31% of stage 9 egg chambers showed a weak, and 36% a strong, *osk* mRNA mislocalization. B, Of *cni*^{AR55}/*cni*^{AA12} mutant egg chambers, 46.5% show L53b staining at the posterior (3.25 cells on average); in 32.8% of stage 9 oocytes, *osk* mRNA is mislocalized to the middle.

METHODS. *cni* mutant egg chambers were *cni*^{AR55}/*cni*^{AA12} (ref. 36); *orb* mutant egg chambers were *orb*^{mel}/*orb*^{F343} (refs 33, 34). Other strains used are: *capu*^{G7}, *K10*¹, *rl*^{10A}, *top*^{CA}, *top*^{CO}, *top*^{QY1}, *spir*^{RP} (refs 15, 30, 32, 35).

genes involved in DV axis formation might also participate in this process. We therefore examined the expression of the anterior follicle cell markers, *L53b* or *slbo*, in: (1) *cappuccino*, *spire* and *orb* mutants which, like *grk*, affect the polarity of both axes^{32,34}; (2) *K10* mutants, which cause a dorsalized phenotype

as a result of the failure to localize *grk* mRNA to the dorsal side of the oocyte nucleus^{14,35}; and (3) the ventralizing mutant *cornichon* (*cni*)^{36,37}. Whereas mutations in *cappuccino*, *spire*, *orb* and *K10* do not affect posterior follicle cell determination, mutants in *cni* result in very similar AP defects to those seen in *grk*⁻

FIG. 5 Mislocalization of the germinal vesicle in *grk* and *top/DER* mutant egg chambers. A, B Hoechst staining to visualize DNA. C, D, *grk* mRNA localization. A, The germinal vesicle lies at the dorsal-anterior corner of wild-type stage 8 and older oocytes. B, In 31% of stage 8 or older *grk*⁻ oocytes, the germinal vesicle lies at the posterior. This phenotype is seen in 5–10% of *top^{ovi}/top^{co}* egg chambers. C, In wild-type stage 9 oocytes, *grk* mRNA localizes above the germinal vesicle in the dorsal-anterior corner. D, When the germinal vesicle fails to migrate, as in this *top^{ovi}/top^{co}* oocyte, *grk* mRNA remains localized to the posterior pole.

METHODS. To visualize DNA, we used the DNA dye Hoechst as described².

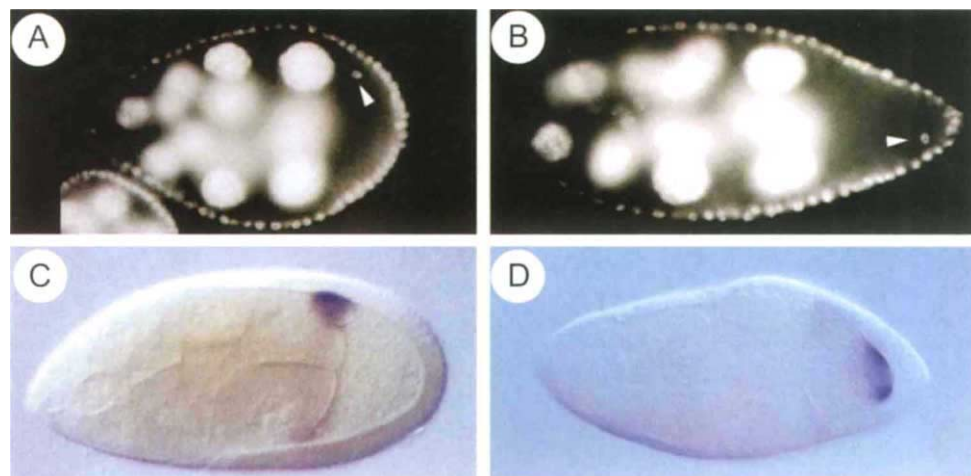
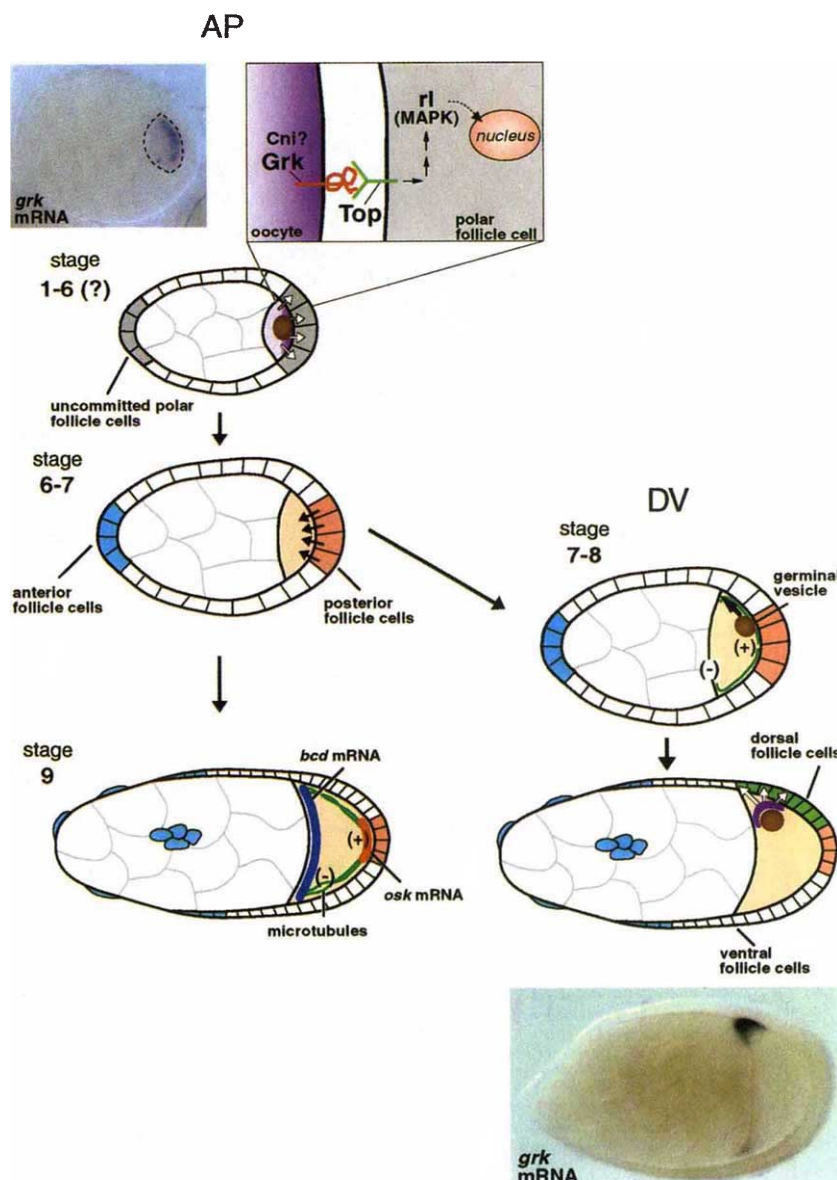


FIG. 6 A model for the generation of AP and DV polarity in *Drosophila*. AP polarity arises from the movement of the oocyte to the posterior of the nurse cells². The germinal vesicle and *grk* mRNA both become localized to the posterior of the oocyte, leading to the polarized production of extracellular Gurken. This then binds to Top/DER in the adjacent uncommitted polar follicle cells to activate a receptor tyrosine kinase signalling pathway to determine posterior follicle cell fate. The posterior follicle cells subsequently signal back to the oocyte to repolarize the oocyte microtubule cytoskeleton, probably by inducing the disassembly of the microtubule organizing centre at the posterior of stage 6 oocytes⁵. The generation of a new microtubule nucleating activity at the anterior margin of the oocyte thus creates a polarized microtubule network, which directs the localization of *bcd* and *osk* mRNAs to opposite poles of the oocyte, thereby defining the AP axis of the embryo. This repolarization of the oocyte cytoskeleton also determines the orientation of the DV axis, because it is required for the microtubule-dependent movement of the germinal vesicle to a site at the anterior margin of the oocyte^{5,38}. The position of the germinal vesicle directs the subsequent relocalization of *grk* mRNA to the dorsal anterior corner of the oocyte¹⁴. The resulting localized synthesis of Gurken induces the follicle cells on this side of the egg chamber to adopt a dorsal fate, as they migrate from the nurse cells to cover the oocyte. Thus the sequential induction of posterior and dorsal follicle cell fates by localized Gurken establishes first AP and then DV polarity.



egg chambers. In nearly half of *cni* mutant egg chambers, L53b is expressed in the posterior follicle cells and *osk* mRNA is mislocalized to the centre of the oocyte (Fig. 4B). Thus, like *grk* and *top/DER*, *cni* is required for the induction of both posterior and dorsal follicle cell fates. Because the ventralized phenotype of *cni* mutants is germline dependent³⁷, Cornichon is probably required in the oocyte for the production of the inducing signal. However, *cni* mutants do not affect the localization of *grk* mRNA to the posterior of the oocyte (data not shown), suggesting that it must act at a different step in this pathway.

AP is the primary axis

During stages 7 and 8 of oogenesis, the germinal vesicle migrates from the posterior of the oocyte to the anterior margin of the cell, in a process that requires microtubules^{5,38}. Because the absence of posterior follicle cells in *grk* and *top/DER* mutants prevents the polarization of the oocyte microtubule cytoskeleton, we examined whether these mutants also affect the positioning of the germinal vesicle. In about one-third of *grk* (or 5 to 10% of *top/DER*) mutant egg chambers, the germinal vesicle fails to migrate to the anterior margin (Fig. 5A, B). This result is most easily explained if the germinal vesicle moves towards the 'minus' ends of the microtubules, which lie at the anterior pole in wild-type oocytes. In the mutant egg chambers where the cytoskeleton develops a symmetric organization, with the 'minus' ends of the microtubules at both poles, the germinal vesicle can localize to either end of the oocyte.

In a wild-type egg chamber, *grk* mRNA accumulates above the germinal vesicle after it has migrated to the anterior of the oocyte¹⁴. In the *top/DER* mutant egg chambers in which the germinal vesicle has failed to migrate, *grk* mRNA also remains localized to the posterior pole of the oocyte (Fig. 5C, D). This suggests that the germinal vesicle directs the localization of the mRNA. As the localization of *grk* mRNA serves to restrict where Gurken signalling induces dorsal follicle cell fate, the orientation of the DV axis is determined by the position of the germinal vesicle, which in turn requires the polarization of the oocyte cytoskeleton that is induced by the posterior follicle cells. Thus the formation of the DV axis depends on the prior polarization of the AP axis.

Discussion

We have previously proposed that AP polarity in *Drosophila* arises when the oocyte moves to the posterior of the egg chamber and induces the adjacent follicle cells to adopt a posterior fate². The results presented here confirm this view, and lead us to propose the model for AP axis formation shown in Fig. 6. One surprising aspect of these results is the discovery that Gurken,

Top/DER and Cornichon are required for the induction of both the posterior and dorsal follicle cells, and hence for the polarization of both the AP and DV axes. This raises the question of how the same signalling pathway can induce two different follicle cell states. Several enhancer trap lines and antibodies label a subset of the follicle cells at each pole of the egg chamber from early oogenesis onwards, suggesting that the polar follicle cells have been specified as distinct from the rest of the follicle cell population before either induction takes place^{3,22,39,40}. It therefore seems likely that the two follicle cell populations differ in their competence to respond to the inductive signal: the fate of the polar follicle cells is restricted to a choice between anterior or posterior, and the rest of the follicle cells are committed to become dorsal or ventral. If this is correct, the orientation of the AP axis is determined by the position of the polar follicle cells. The inductive signal from the oocyte then imposes polarity on this axis by directing the cells at one end of the egg chamber to adopt a posterior fate.

Because the AP and DV axes are independent later in oogenesis and during early embryogenesis²⁰, the relative orientation of the two axes is determined by the positions of the posterior and dorsal follicle cells. These depend on the placement of the germinal vesicle, and the concomitant localization of *grk* mRNA, first to the posterior of the oocyte and then to the dorsal anterior corner. Our results indicate that the two axes are not independent at this stage, as the polarization of the oocyte microtubule cytoskeleton along the AP axis directs the movement of the germinal vesicle to the anterior of the oocyte (Fig. 6). Thus AP is the primary axis in *Drosophila*, and DV the secondary. Indeed, it is the anterior movement of the germinal vesicle and *grk* mRNA that is responsible for the orthogonal orientation of the two axes.

It is hard to imagine how two perpendicular axes can be defined unless the orientation of one depends on the other. In this context, it is interesting to note that there is evidence that DV axis formation in *Xenopus* also depends on the prior establishment of the primary axis. The animal-vegetal axis forms during oogenesis, leading to the localization of the germ plasm and several maternal mRNAs at the vegetal pole of the oocyte⁴¹. In the freshly laid egg, the vegetal cytoplasm contains an unidentified, but transplantable, dorsalizing activity⁴². After fertilization, a microtubule-dependent process associated with cortical rotation moves this activity from the vegetal pole of the egg to a site in the equatorial region, where it is thought to induce DV polarity^{42,43}. Thus it may be that, in *Xenopus* as in *Drosophila*, a dorsalizing signal moves from the site of germ plasm formation to the future dorsal side, thereby defining a second axis that is perpendicular to the first. □

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Gravitationally redshifted emission implying an accretion disk and massive black hole in the active galaxy MCG-6-30-15

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ACTIVE galactic nuclei and quasars are probably powered by the accretion of gas onto a supermassive black hole at the centre of the host galaxy¹, but direct confirmation of the presence of a black hole is hard to obtain. As the gas nears the event horizon, its velocity should approach the speed of light; the resulting relativistic effects, and a gravitational redshift arising from the proximity to the black hole, should be observable, allowing us to test specific predictions of the models with the observations. Here we report the detection of these relativistic effects in an X-ray emission line (the K α line) from ionized iron in the galaxy MCG-6-30-15. The line is extremely broad, corresponding to a velocity of $\sim 100,000 \text{ km s}^{-1}$, and asymmetric, with most of the line flux being redshifted. These features indicate that the line most probably arises in a region between three and ten Schwarzschild radii from the centre, so that we are observing the innermost region of the accretion disk.

Recent optical and radio observations²⁻⁴ of gas motions show a large, central concentration of mass (probably a black hole) in a number of galaxies. But this gas lies beyond $\sim 30,000$ Schwarzschild radii so the characteristic signatures of the black hole itself—relativistic effects due to its strong gravitational field—were not observed. X-rays are produced much closer to the black hole and provide better access to these effects, particularly if a spectral line is emitted for which energy shifts can be clearly measured.

An iron K α emission line close to 6.4 keV and an additional, hard-continuum component are imprinted on the primary, power-law, X-ray continuum of most Seyfert 1 active galactic nuclei (AGN)^{5,6}. They arise via fluorescence and back-scattering ('reflection') from optically thick material which covers about half the sky seen by the primary continuum source^{7,8}. The data do not allow the geometry of the material to be determined unambiguously, but are consistent with an irradiated accretion disk⁹⁻¹². The emission lines from such a system are expected to be very broad, as most fluorescence occurs in a regime where Doppler and gravitational shifts are large^{9,12,13}. Clear evidence for significant broadening—at a level compatible with accretion-disk models—has now come from new data (from the ASCA satellite) on several AGN, including MCG-6-30-15^{14,15}. The emission lines should also have a characteristic profile. The strongest lines are produced in a face-on geometry¹⁰, where gravitational and transverse Doppler effects dominate, producing a profile skewed to the red. For edge-on disks the line is weaker, and the blue side of the profile (the blue 'wing') more prominent^{9,16}.

MCG-6-30-15 was observed by ASCA¹⁷ on 23 July 1994 for approximately four days, with both the CCD (charge-coupled device) detectors (SIS) and gas-scintillation proportional counters (GIS) in operation. Here we present only the SIS data, which have the better energy resolution; the GIS and SIS data are entirely consistent. A complicating factor in the analysis of the integrated SIS spectra is the presence of features from highly ionized gas in the line of sight, the so-called warm absorber¹⁸, which is now well established in this source^{14,19,20}. Initially, then, we fitted the spectra in the range 0.4–10 keV with a model consisting of a power law emitter and photoionized absorber. This shows that the absorption only affects the spectrum below 2 keV, and therefore in analysing the line data we have restricted the energy range to 2.5–10 keV. Previous data from the Ginga satellite also showed⁶ strong evidence for a reflection continuum component accompanying the iron-emission line. We have assumed a face-on slab subtending 2π sr at the X-ray source and calculated the reflection spectrum using the model of Lightman and White⁸. As expected from previous observations⁶, the spectrum and residuals for the 3–10 keV continuum fit show a well defined excess in the residuals at ~ 6 keV (Fig. 1), most probably an iron K α line, but much broader than the instrument resolution for a

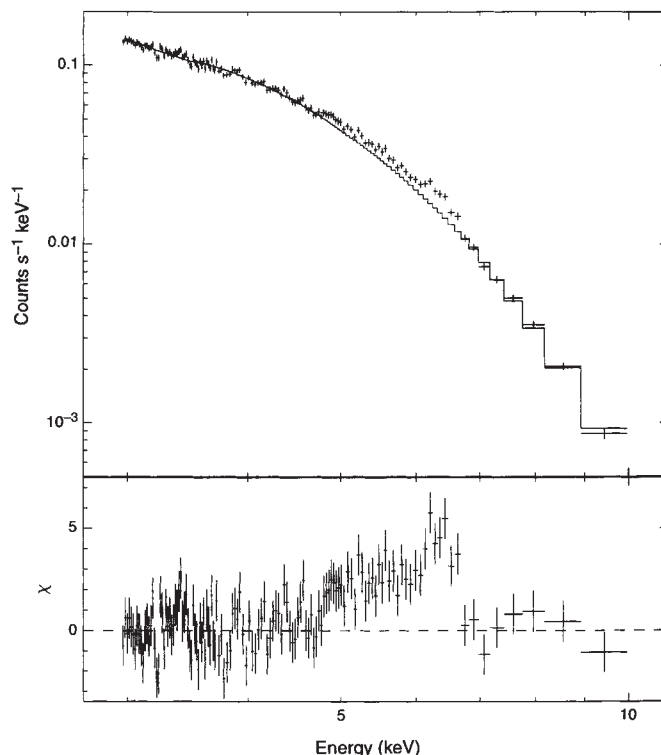


FIG. 1 X-ray spectrum of MCG-6-30-15, as observed by the ASCA satellite using the SIS detectors. Top panel, observed spectrum (crosses) and fitted 'power-law plus continuum reflection' model (stepped line; the model has the same energy bins as the data). This fit excludes data between 5 and 7 keV. Bottom panel, data-minus-model residuals. The emission line is clearly visible with an asymmetry to the red. Parametrizing the observed emission line with a single gaussian improved the fit by $\Delta\chi^2=82$ with a centroid energy of $E_K=5.92^{+0.16}_{-0.15}$ keV, width $\sigma=0.74^{+0.24}_{-0.18}$ keV and equivalent width $EW=330^{+180}_{-120}$ eV. The power-law continuum has photon index $\Gamma=2.05\pm0.07$. Adding an additional gaussian improved the fit further, with $\Delta\chi^2=39$, to $\chi^2=656.0$ (675 degrees of freedom). The double-gaussian parametrization consists of a relatively narrow ($\sigma=0.18$ keV) component at 6.4 keV and a broader ($\sigma=0.64$ keV) wing at ~ 5.5 keV. The red wing carries more flux than the core, with equivalent widths of 200 eV and 120 eV respectively.

single narrow line. We emphasize that the statistical quality of the data is sufficient to allow the precise determination of the continuum above and below the line; the derived line profile depends little on the uncertainty in the continuum shape, or the normalization of the reflection continuum.

It is clear from the residuals (Fig. 1) that the line profile is not gaussian. It consists of a relatively narrow core around 6.4 keV with a very broad wing extending to the red. We have modelled the line with two gaussians, one each for the core and wing (see Fig. 1 legend for details). This fit allows us to determine the best-fit model for the continuum. The deviations of the data from this continuum model represent the line profile, which is shown in Fig. 2. The data have been rebinned to approximately the instrumental resolution and are shown in incident (photon) flux. The profile is clearly extremely broad and asymmetric. Most of the flux is strongly redshifted from the rest energy of even the lowest-energy K α lines (6.4 keV), by as much as $\Delta E/E \approx 0.3$. The full width at zero intensity corresponds to a velocity of the order of 100,000 km s⁻¹.

From the large redshift, we can immediately reject the hypothesis that Compton scattering produces the observed line profile²¹. Such scattering requires that energy is transferred from the photons to the electrons and thus that $kT < 1.6$ keV in any scattering plasma ($4kT < \text{photon energy}$). The energy shift per scattering is small (~ 80 eV) and a high Thomson optical depth (~ 5) is required for the scattering plasma to produce the total shift in the line centroid. Such a plasma would also Compton scatter the continuum photons to lower energies, creating a break at around 20 keV. No such break is observed²².

The Doppler width of the line implies velocities of the order of $0.3c$ (where c is the velocity of light), indicating highly relativistic motions. The line flux drops sharply above the rest energy of 6.4 keV, which excludes any velocity distribution where substantial amounts of the material is moving towards us. This would cause enhancement of the blue wing, which is not observed. Symmetrical outflows and spherical or quasi-spherical distributions of orbiting clouds therefore cannot account for the observed profile. The observation of broad redshifted lines in several objects¹⁵, but no strong blue-shifted lines, argues against any asymmetrical-outflow hypothesis in which the flow is directed away from us (some objects should then have the flow directed towards us). It is therefore most probable that the material is moving primarily transversely at a large fraction of the speed of light with the width of the line being produced by transverse Doppler and gravitational redshifts close to a central

black hole. The profile seems to be remarkably similar to that predicted for a face-on accretion disk^{9,12}.

To determine whether our line profile can be accounted for by a black hole and accretion disk, we initially employed a model which assumes a disk orbiting a Schwarzschild black hole⁹. The free parameters in this model are the inclination of the disk, i , its inner and outer radii, R_i and R_o , and the index of the emissivity function, α ; the line emissivity is assumed to vary as $R^{-\alpha}$. Initially, we assumed a rest energy of 6.4 keV appropriate to species less ionized than Fe xvi (more highly ionized species are discussed later). The model gives $\chi^2 = 655.7$ (676 degrees of freedom), very similar to the double-gaussian model, but with one fewer free parameter. The best-fitting value is $\alpha = 0.7$, but is not well determined. For a centrally illuminated disk, we expect a rather flat emissivity profile in the central regions, $\alpha \approx 1$ increasing to $\alpha \approx 3$ in the outer disk¹⁰. We have fixed α at these two values, which both fall within the 68% confidence region in the fit, to determine the other parameters. For $\alpha = 1$, we find $i = 30.2^{+1.5}_{-2.7}$, $R_i = 3.4^{+3.0}_{-0.4} R_s$ and $R_o = 7.4^{+2.5}_{-0.8} R_s$, with equivalent width $EW = 390^{+90}_{-130}$ eV. For $\alpha = 3$, the parameters are $i = 29.7^{+2.9}_{-3.9}$, $R_i = 3.7^{+1.8}_{-0.7} R_s$, $R_o = 10.0^{+12.5}_{-3.2} R_s$ and $EW = 380^{+100}_{-110}$ eV, very similar to those for $\alpha = 1$. Note that the minimum value for R_i in the Schwarzschild geometry is $3R_s$, so the lower error bar on this value is not statistical. In the Kerr metric of a spinning black hole the last stable orbit is closer to the central hole. We have also tested this condition¹⁹, for which the parameters, $i = 26.8^{+2.1}_{-1.0}$, $R_i = 4.7^{+0.83}_{-0.9} R_s$, $R_o = 16.7^{+7.6}_{-7.6} R_s$, $\alpha = 4.5^{+3.9}_{-3.7}$ and $EW = 300^{+70}_{-50}$ eV, and χ^2 are very similar to those of the Schwarzschild black hole. The uncertainties in the parameter values depend on the assumptions inherent in the models. For example, the value of R_o does not necessarily represent the true outer radius of the accretion disk, but is simply that radius beyond which no significant iron K α line emission occurs. The rather low value derived in our fits implies that the emission line is produced very near to the central black hole and that the primary X-rays originate close to the surface of the inner disk.

An iron-line equivalent width of 250–400 eV is a factor of ~ 2 –3 higher than the predictions of simple disk reflection models^{10,11}. There are several possible explanations for this. First, a modest increase of a factor ~ 1.5 –3 in the iron abundance relative to the lighter elements would produce a line flux compatible with that reported here^{10,23}, the primary uncertainty being the assumed cosmic abundances. Another possibility is that there is another source of line emission. For example, there may be a contribution to the emission line from a molecular torus^{24–26} surrounding

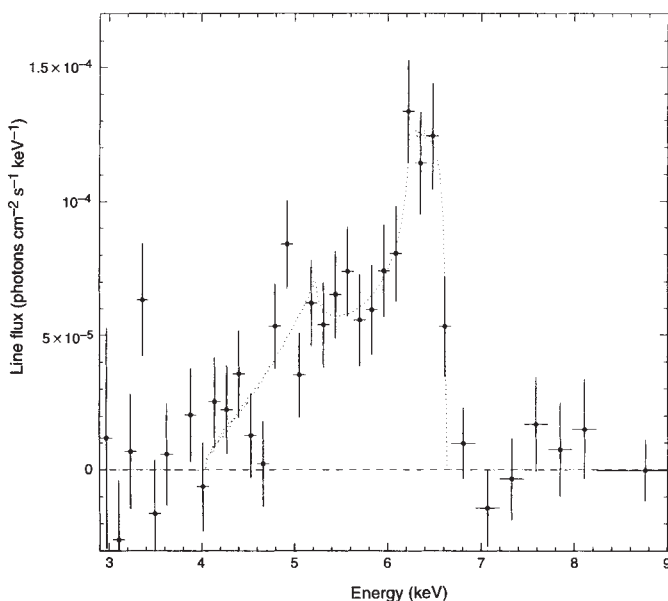


FIG. 2 The line profile of iron K α in the X-ray emission from MCG-6-30-15. The data have been rebinned to approximately the instrumental resolution. The emission line is very broad, with full width at zero intensity of $\sim 100,000$ km s⁻¹. There is a marked asymmetry at energies lower than the rest-energy of the emission line (6.35 keV at the source redshift of 0.008). The most plausible mechanisms which can produce this extensive red wing are transverse Doppler and gravitational shifts close to a central black hole. The dotted line shows the best-fit line profile from the model of Fabian *et al.*⁹, an externally-illuminated accretion disk orbiting a Schwarzschild black hole.

the nucleus at a distance of ~ 1 pc. Because of this large distance we expect the K α line to be narrow compared to the instrumental resolution and centred at 6.4 keV in the rest frame. The addition of a narrow (dispersion $\sigma = 10$ eV) gaussian at 6.4 keV to our 'power-law plus reflection continuum' model gives an equivalent width of only ~ 45 eV. This is a conservative upper limit to the torus contribution, because it allows no emission at 6.4 keV from the disk line. A large contribution from a torus is therefore unlikely, and in any case does not change our conclusions regarding the remainder of the line. A final possibility is that the material is highly ionized^{27,28}. An increase in the effective fluorescence yield and reduced photoelectric opacity between 6 and 7 keV would then result in a higher line flux, provided that a large proportion of the iron is in the helium-like and hydrogen-like states. Such a scheme is compatible with our data; the

increase in rest energy to 6.7–6.9 keV can be compensated for by a smaller inclination angle and/or concentrating the emission closer to the black hole, thereby increasing the gravitational shift.

Given the strong evidence for the black-hole/accretion-disk model in MCG–6–30–15, we infer that the broad lines observed in other AGN (even though less well-resolved) are produced by the same process. Modelling these profiles can reveal extraordinary detail about the central regions of the AGN, as illustrated by the disk line fit above. We already have indications that the X-rays are produced very close to the surface of the disk, and close to the black hole. Observations of variability of the emission line, and its time-lag with respect to variations in the intensity of the continuum emission may allow us to measure the black hole mass and spin^{9,29}. $\square$

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A massive counter-rotating gas disk in a spiral galaxy

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In some galaxies that have little gas remaining from the epoch of galaxy formation, the gas that is present rotates in the opposite sense to the stars^{1–4}. It is thought that this counter-rotating gas may result from the capture by a massive early-type galaxy of a gas-rich dwarf galaxy that was orbiting in the opposite sense to the main galaxy's rotation; but as there are few examples of galaxies with counter-rotating gas, we have little information about the details of this process. Here we present optical spectra of the spiral^{5,6} galaxy NGC3626, which clearly show the presence of counter-rotating ionized gas; combining these results with pre-existing atomic hydrogen data⁷ leads to an estimate of $10^9 M_\odot$ for the mass of the gas. Spiral galaxies generally contain substantial amounts of gas left over from the formation epoch, which would be co-rotating with the stars; this gas should interact strongly with the counter-rotating gas on a relatively short timescale, causing it to fall towards the centre of the galaxy. We therefore propose that the counter-rotating gas in NGC3626 has been captured recently; the merger process is just beginning.

We observed NGC3626 in February 1992 and March 1994 at the 1.82-m Asiago-Ekar telescope, using the Boller and Chivens spectrograph at a dispersion of $1.9 \text{ \AA}$ per pixel and a resolution of 1.16 arcsec per pixel. These spectra cover two wavelength ranges: $4,800\text{--}5,800 \text{ \AA}$ with the slit at position angle $PA = 158^\circ$ (the major axis of the stellar component) and at $PA = 113^\circ$ and $3,700\text{--}4,760 \text{ \AA}$, with the slit set at $PA = 25^\circ$ and 68° . We obtained an additional spectrum at $PA = 158^\circ$ in March 1995 with the

same telescope and the same resolution but at higher dispersion ($0.96 \text{ \AA}$ per pixel) and covering $6,300\text{--}6,800 \text{ \AA}$. The exposure time for each spectrum was one hour.

Absorption-line velocities, which give the motions of the stars, were measured by means of the Fourier Quotient software package (developed by D.B. and G.G. at Padua Observatory) using the spectra of giant K stars as zero-velocity dispersion templates. The H α absorption and all the emission lines were measured by fitting gaussians over a linear continuum around each line. The motion of the gas was determined using emission lines from the regions of ionized gas surrounding massive young stars (H II regions). We measured the [O II] $3,727\text{--}29 \text{ \AA}$ emission (the line was unresolved), [O III] $5,007 \text{ \AA}$, H α , [N II] $6,548\text{--}6,584 \text{ \AA}$ and [S II] $6,717\text{--}6,731 \text{ \AA}$. (The [O III] lines at $PA = 113^\circ$ are too faint with respect to the continuum after sky subtraction to fit a profile.) The automatic fit from the data-reduction software was checked manually: every line from the CCD (charge-coupled device) detector was displayed on the screen, the cursor pointed on the line, and after the peak and the continuum were identified, a gaussian fit was determined.

The emission and absorption lines have opposite inclinations in the spectra, which indicates that the stars and ionized gas (Fig. 1) are moving in opposite directions from the nucleus out to an angular distance of 20 arcsec . There, both the gas and stars reach mean observed velocities of $\sim 160 \text{ km s}^{-1}$ with respect to the nucleus (but in opposite directions), indicating that both components are moving under the influence of the same gravitational potential. The gas counter-rotation seems to be present both along the major axis of the galaxy ($PA = 158^\circ$) and at 47° from it ($PA = 205^\circ$). There is no evidence for stellar counter-rotation at the limit of the instrumental resolution. Along the apparent minor axis ($PA = 68^\circ$) the gas and the stars show essentially zero velocity differential from the velocity of the galaxy nucleus (within $\sim 48 \text{ km s}^{-1}$; Fig. 1), as expected for regular motions within the potential well of the galaxy.

With the exception of the major axis, the velocity dispersion curves for the stars are confined to the innermost $\pm 15 \text{ arcsec}$.

They reach a maximum of $\sim 200 \text{ km s}^{-1}$ in the central regions, and then decrease to 150 km s^{-1} (Fig. 1), a value typical of stars in 'normal' early spiral galaxies⁸. This suggests that the stellar component is not dynamically hotter than in other galaxies of the same type.

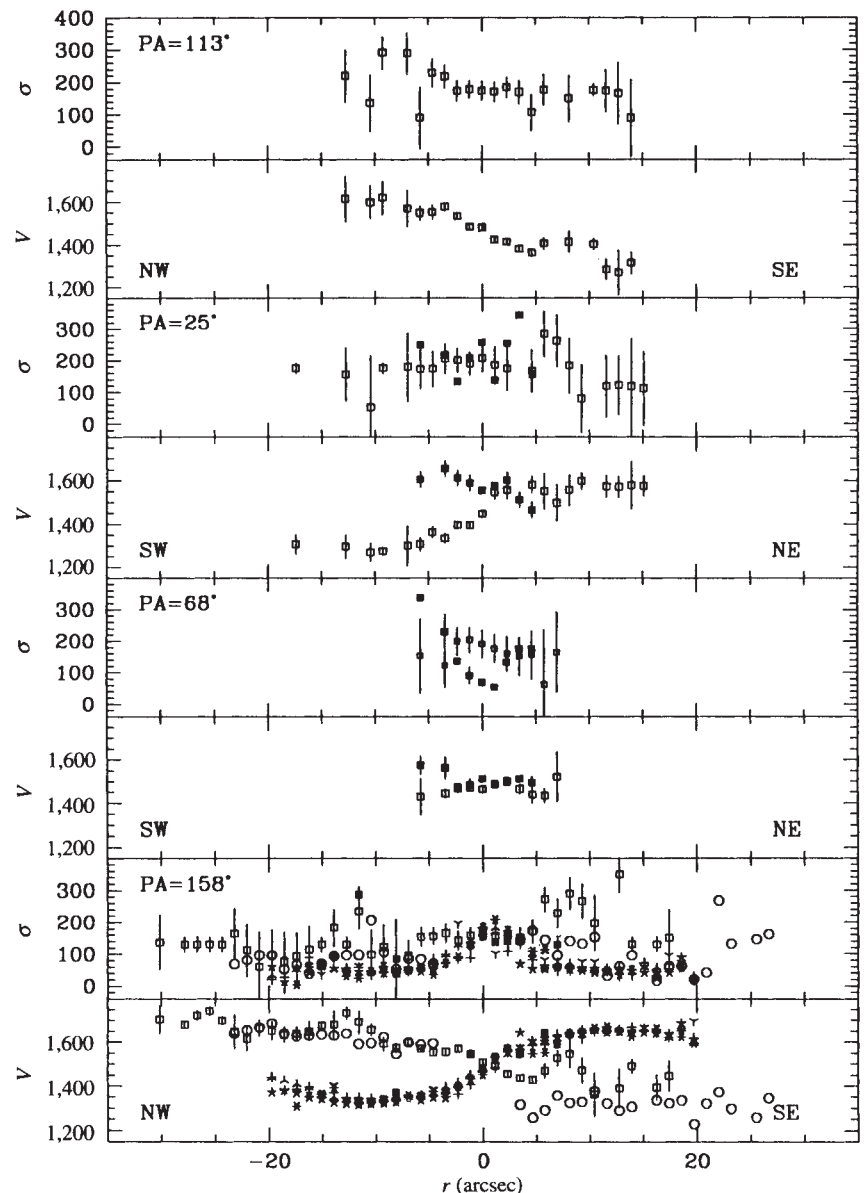
The stellar velocity field shows a marked symmetry with respect to the major axis. The zero-velocity line lies near the apparent minor axis (Fig. 1), and the velocity curves at $+47^\circ$ and -45° from the major axis show similar amplitudes. However, the observed velocities at these intermediate position angles are consistent with stars streaming not on circular, but on elliptical, orbits (with ellipticity $\epsilon=0.3$); it is not clear from our data if this can be attributed to the influence of the inner lens-like structure visible in the images⁹.

At the assumed⁹ distance (20.8 Mpc) and inclination ($i=46^\circ$) our deprojected rotation curve for the stars extends 3 kpc from the centre, with the outermost values oscillating around $\sim 300 \text{ km s}^{-1}$ (and the northwestern side receding). The direction of the stellar rotation outside 3 kpc could be deduced by means of the two faint (possibly stellar) spiral arms⁹ visible out to 2 arcmin ($\approx 12 \text{ kpc}$) from the centre in the Palomar Sky Survey

and in our CCD frames (taken at the 1.82-m Asiago-Ekar telescope). The images indicate that the spiral arms are trailing with respect to the stellar rotation, as expected in a normal spiral galaxy. The stellar rotation may extend to a radial distance of 12 kpc, with the northwestern side receding.

On the other hand, the gas motions may be consistent with circular rotation, with an amplitude of $\sim 200 \text{ km s}^{-1}$ at 2 kpc. When we add to our data the 21-cm observations present in the literature^{7,9}, we find that the atomic hydrogen counter-rotates with respect to the stars; it rotates in the same sense as the ionized gas (northwest approaching⁹). The H I data cover the region between ~ 15 and 20 kpc from centre⁷, and show similar rotational velocities: $\sim 200 \text{ km s}^{-1}$. The H I distribution suggests an irregular structure, with a concentration 15 arcsec east of the nucleus and an inner structure elongated near the minor axis⁹. A clumpy ring structure with an extent of $80 \times 120 \text{ arcsec}$ (elongated along $\text{PA} = 165^\circ$ (near the major axis)) is connected to an outer distribution of H I elongated in a north-south direction. The average radial distribution of H I, however, for NGC3626 is described⁷ as similar to a filled, exponential disk, rather than a ring-like configuration.

FIG. 1 The observed velocity curves for NGC3626 at four position angles. For each angle, the velocity values V and the velocity dispersion σ , both in km s^{-1} , are plotted against the distance r from the galaxy nucleus. The major axis of the galaxy is at position angle $\text{PA}=158^\circ$. Data from the Fourier Quotient software are plotted as open squares, while the H α absorption data are shown as open circles. Emission lines are plotted as star-type symbols when measured from H α ($\star$), [N II] 6,548–6,584 Å ($\times$ and λ) and [S II] 6,717–6,731 ($+$ and Y), and filled squares when measured from the [O III] 5,007 Å ($\text{PA}=158^\circ$) and [O II] doublet ($\text{PA}=25^\circ$ and 68°). The presence of an obscuring dust arc^{9,31} is responsible for the noisy data at $\sim 10 \text{ arcsec}$ SE along the major axis and $\sim 10 \text{ arcsec}$ NE along $\text{PA}=25^\circ$. The X-shaped appearance of the velocity curve at $\text{PA}=158^\circ$ is typical of galaxies showing extended counter-rotation, with stars on the northwestern side receding and gas approaching. All velocities are heliocentric, with a systemic mean value equal to $1,479 \pm 27 \text{ km s}^{-1}$. The spectra were reduced (using the IRAF software) by subtracting the bias and dark frames, then flat-fielding with frames exposed using a continuum lamp. The sky spectrum was subtracted using 20 lines on the same galaxy spectrum at the opposite edges of the slit, where the continuum is negligible.



Despite the lack of data between 2 kpc (the outer limit of our emission-line data) and 15 kpc (the innermost reliable value from H I; ref. 7), the correspondence in both sense and magnitude of rotation for the ionized and atomic gas, as well as the disk-like H I radial distribution, suggests strongly to us that the counter-rotation with respect to the stars extends across the entire range. The total H I mass detected⁷ in NGC3626 is $(0.87 \times 10^9) M_{\odot}$; it may be confined to clumpy regions and lie mainly in the outer part of the galaxy.

We see in NGC3626 many of the peculiarities that are only partially present in other systems: the gas counter-rotates with respect to stars at both small and large radii, the mass of this counter-rotating gas is quite high ($\sim 10^9 M_{\odot}$) and its morphology is that of a spiral galaxy.

The last point is important because the capture of a small, gas-rich donor should proceed along different evolutionary paths according to whether the acceptor galaxy is gas-poor (E or S0) or gas-rich. Gas entering an already gas-rich system, such as a spiral galaxy, should undergo numerous collisions with pre-existing clouds which, through the cancellation of angular momentum, would make the gas collapse towards the nucleus in very short time. This gas could be expected to form new stars, or to evolve in complex ways¹⁰⁻¹³. This would seem to make the observation of spirals with gas and stars in counter-rotation rather exceptional.

In S0 galaxies, gas counter-rotating across a wide range of radii has been already observed in NGC4546^{3,14}, while in NGC4550 $\sim 50\%$ of the stars are counter-rotating^{15,16}. In spiral galaxies, the only case of gas-rotation known is in the 'black-eye' galaxy NGC4826^{10,17-19} which shows only a region of partial counter-rotation. There, the inner gas is co-rotating and only an outer H I ring is in counter-rotation. In NGC7217²⁰, another spiral galaxy, the gas is co-rotating with most of the stars, but about 20–30% of the stars are counter-rotating. In all the above cases of gas counter-rotation, however, the quantity of gas involved is at least an order of magnitude lower than in NGC3626. On the other hand, the total masses of counter-rotating stars^{21,22} are very high, of the order of $10^{10} M_{\odot}$. We suggest that NGC3626, by collecting together all the above properties, could be the evolutionary link between the various types of counter-rotation, indicating that this phenomenon happens for all morphological types and at all scales of dimension and masses.

As for similar cases of counter-rotation in S0 galaxies^{2,3,14} we must assume that this counter-rotating gas has been accreted from outside. This involves the acquisition and destruction of a donor source (which may be a satellite galaxy), whose stellar component (if present) is smeared inside the acceptor galaxy and mixed with its stars, while the gas from the donor becomes distributed in a ring or a disk which evolves independently of the acceptor galaxy. The impact angle (of the collision between the donor and acceptor) determines the final spin of the accreted material: aligned (co-rotating), perpendicular to, or opposite that of the galaxy. The two last cases are what we call polar ring galaxies²³⁻²⁵, and disks with counter-rotation¹⁻⁴. Whatever the donor source, if the acceptor galaxy is much more massive, the presence of the accreted stars will be unnoticeable among the greater numbers of stars in the acceptor unless the following occur: (1) the compact nucleus of the donor survives intact in the centre of the acceptor, which might be the case for elliptical galaxies with counter-rotating stellar cores^{26,27}; (2) the donor is mostly composed of stars and is massive enough to generate distinct absorption lines (which may explain the S0 galaxy NGC4550^{15,16} and the Sb galaxy NGC7217²⁰); (3) the counter-rotating gas has time to settle inside the galaxy (as in the SB0 galaxy NGC4546^{3,14}) and later forms stars (as above, NGC4550 and NGC7217). The accreted gas, unlike the stars, can easily be traced inside the acceptor galaxy by means of its emission lines (optical, H I or CO).

It is often implicitly assumed that the donor is small compared with the acceptor, a condition required by the complex and

undisturbed stellar structure of these disk (sometimes barred-disk) galaxies. In agreement with this, all the systems with counter-rotation have a gas mass between 0.3% and 1.4% of the total mass²². Donors with progressively bigger masses, colliding in counter-rotating orbits, would heat the stellar disk more and more until the extreme event of equally massive donor and acceptor, in which case the galaxies merge together, thereby losing their original morphology (for example, NGC7252²⁸). The full merging could generate counter-rotation, but only for elliptical galaxies²⁹.

In cases of 'massive' counter-rotation in disk galaxies, the only alternative seems to be the slow accretion of stars and/or diffuse gas with opposite spin. Models³⁰ show that the slow infalling matter and the disk galaxy can realign without the formation of highly inclined rings; instead, coplanar structures are formed.

In the case of NGC3626, the quantity of counter-rotating material is so large that it is hard to attribute it to the ingestion of a single dwarf satellite galaxy. The disk velocity dispersion is about half the deprojected rotational velocity, a ratio typical of early spirals⁸, suggesting that the accreted matter has not dynamically heated the disk very much. We note that 5 arcmin south of NGC3626, where the H I gas is moving at 200 km s^{-1} with respect to the galaxy nucleus, there is a companion galaxy, UGC6341, which is moving with a relative velocity of $+160 \text{ km s}^{-1}$. This satellite galaxy cannot yet be the donor source, because it is already gas-rich (14% of its total mass) and no H I bridges have been detected between the two galaxies⁹. The donor source for NGC3626, if a satellite galaxy or a dark cloud, should be three times more massive than UGC6341. Two possibilities remain: (1) we are observing the beginning of a merging phenomenon between NGC3626 and a massive gas (gas-rich?) companion that will heat the disk or eventually disrupt both galaxies, or (2) this case is the result of a slow infall of gas from the surroundings, which may be destined to form stars and ultimately to produce a system like NGC4550, with two counter-rotating structures in the same plane. $\square$

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Purification of molecular mixtures below the eutectic by emulsion crystallization

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MELT crystallization¹ is used increasingly by the chemical industry to purify organic compounds, in part because of the availability of new processing techniques² and in part because of the commercial and legislative pressures demanding that such compounds be supplied with precisely defined compositions. At present, purification is generally achieved by a succession of steps involving freezing on a cooled surface followed by partial melting³. As an alternative, we have been examining the process of emulsion crystallization⁴, in which a melt is crystallized within emulsified droplets so that homogeneous nucleation occurs at a lower rate than in a bulk melt. This approach requires no special equipment or solvents, and so offers the possibility of purification at low capital and operating costs. Here we report the use of this method to purify mixtures of *meta* and *para* chloronitrobenzene (*m*- and *p*-CNB) below their eutectic temperature at compositions where bulk crystallization would yield a mixture of pure *m*- or *p*-CNB and a *m/p* combination of the eutectic composition. Adding seed crystals of *m*-CNB to various *m/p* mixtures below the eutectic composition results in the formation of crystals highly enriched in *m*-CNB, while the *p*-CNB remains primarily in the emulsion phase. Thus emulsion crystallization has the potential to 'break' the eutectic, which otherwise presents a barrier to the separation of eutectic-forming mixtures.

The use of emulsified systems as a technique for studying homogeneous nucleation has become well known since the pioneering work of Turnbull⁵ on metals and paraffins. Dispersion of a melt into discrete emulsified droplets allows heteronuclei to be isolated in certain drops and homogeneous nucleation to occur in the remainder. The probability of finding a heteronucleus in a drop decreases with drop size: hence nucleation has been found to be more difficult in smaller drops⁶.

For this study we have chosen to investigate the purification of *meta*-chloronitrobenzene from two-component mixtures with its *para* isomer. This system exhibits eutectic behaviour (Fig. 1),

TABLE 1 The effect of crystallization temperature on final crystal purity

Original droplet composition (<i>m/p</i> CNB)	Liquidus temperature (°C)	Crystallization temperature (°C)	Final* crystal composition (% <i>m</i> -CNB)
90:10	41	30	99.7
		28	99.3
		28†	90.7
		24	98.2
		22	93.6
		22†	90.4
75:25	34	25	99.7
		23	98.4

* After 90 min.

† Equivalent data measured in single-stage progressive freezing onto a cooled surface.

and we have studied *m*-CNB-rich melts, where bulk crystallization results in the formation of pure *m*-CNB and/or the *m/p*-CNB eutectic mixture. There is no evidence of solid-solution behaviour in this system, although previous studies of melt and solution crystallization of *m*-CNB have shown⁷ that the presence of *p*-CNB induces a polar morphology in *m*-CNB crystals.

In the experiments reported here, *m/p* mixtures of 90:10 and 75:25 were emulsified above their melting point, at a volume fraction of 2% in water using the sulphated alkylethoxolate surfactant Tensagex (supplied by Hickson-Manro, Manchester, UK). At an overall concentration of 0.2 wt% Tensagex has no effect on the phase diagram (Fig. 1), and stable oil-in-water emulsions were prepared with drop sizes in the range 5–10 µm. These were then crystallized by cooling at fixed rates. As expected at these small drop sizes, the emulsions showed significant undercooling (typically 30–40 °C). To overcome this problem and perform crystallization at fixed temperatures, a simple seeding technique was used in which the emulsion was cooled to the desired crystallization temperature and 1 mg ml⁻¹ of pure *m*-CNB crystals added. The subsequent progress of crystallization in terms of purity, yield and crystal form was monitored by investigating aliquots with gas chromatography (sensitive down to 0.02% *m*-CNB) and optical microscopy. Table 1 shows, for the two compositions crystallized at various temperatures, the product purity after 90 min compared to equivalent material crystallized in a single stage (with no partial melting) on a cold surface.

A single-step emulsion crystallization clearly offers considerable improvement (in terms of purity) over its progressive-freez-

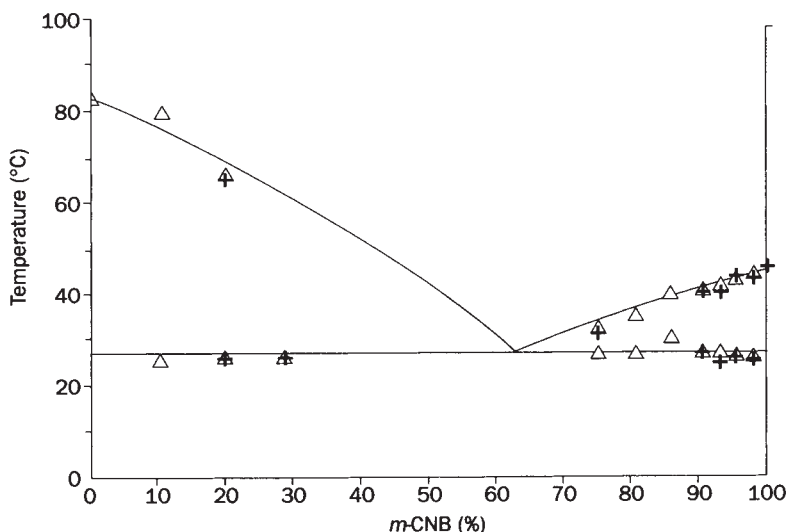
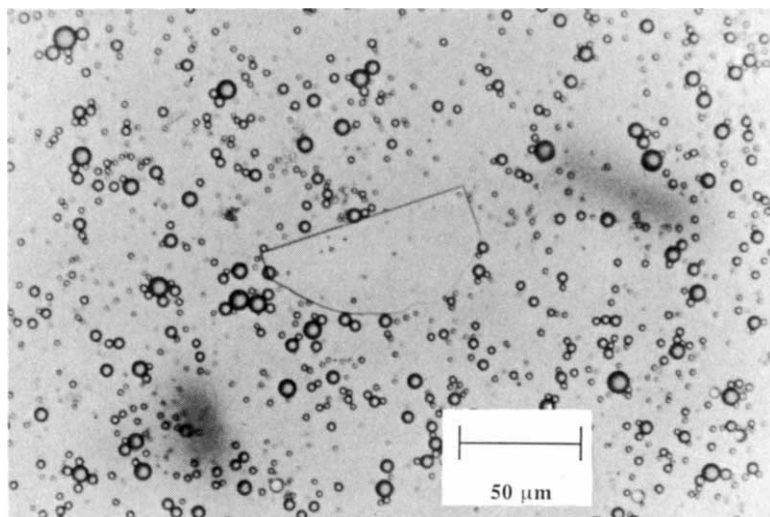


FIG. 1 The experimentally determined *m/p*-CNBN phase diagram, with (+) and without (Δ) 8 wt% Tensagex (added as a 27 wt% aqueous solution). Data were recorded using the Mettler TA 3000 differential scanning calorimeter; the experimental melting points and heats of fusion were respectively 83 °C and 23.9 cal g⁻¹ for *p*-CNB, and 45.5 °C and 30.4 cal g⁻¹ for *m*-CNB. The assumption of ideal behaviour leads to predicted values of 62.9% *m*-CNB and 27.1 °C respectively for the eutectic composition and temperature, as shown by the overlaid curves (—).

FIG. 2 A crystal of *m*-CNB growing from 90:10 *m/p*-CNB emulsion at 22 °C.



ing counterpart. This factor alone suggests that an unusual crystallization process must be operating, as the difficulty of separating crystals from impure melt normally leads to the need for multiple steps and partial melting. This was confirmed by observations of the physical form of the product crystals. First, it was evident that the added seeds acted only to catalyse nucleation of the emulsion, as they remained easily identifiable visually and represented only a small fraction of the final crystal population (5 wt%). Second, and more surprisingly, the growing crystals were always found to be in the aqueous phase and were always single crystals at least an order of magnitude larger than the drop size, with polar morphologies typical of crystals grown from melts or solutions in the presence of *p*-CNB (Fig. 2). This suggests that crystal growth does not occur as a result of crystal-drop collisions, because this would lead to polycrystalline aggregates comprising spheres of similar size to the emulsion drops, with purities close to those obtained on a cooled surface. Overall, however, the most striking result (Table 1) is that at both starting compositions significant purification was still possible at temperatures below the eutectic (27.1 °C), where, according to the phase diagram, crystallization should produce crystals of the *m/p* eutectic composition as well as (for these compositions) crystals of pure *m*-CNB.

These observations may be rationalized as follows. First, the observation that seed crystals initiate crystallization suggests that secondary nucleation⁸, resulting from impacts between the

added seed crystals and between seeds and drops, is the dominant nucleation process. We assume that adsorption of dissolved Tensagex onto the surfaces of these hydrophobic nuclei allows them to be dispersed into the aqueous phase. Second, because at constant crystallization temperature the chemical potential of *m*-CNB will be higher in the liquid phase (the emulsified melt) than in the crystalline phase, it follows that the solubility of liquid *m*-CNB in water is greater than crystalline *m*-CNB. Thus the melt and the aqueous phase will equilibrate to establish a supersaturation relative to the crystalline phase, so the crystals will grow, with transfer of *m*-CNB from the melt droplets to the crystals via the aqueous phase. This allows the growth of large single crystals and means that the growing crystals are not in contact with impure melt, a factor which aids their ultimate separation. The development of a polar morphology suggests that crystal growth is influenced by *p*-CNB, presumably because this isomer also has some solubility in water⁷. Experiments performed below and above the critical micelle concentration of Tensagex (measured in this work to be 0.02 wt% in water at 25 °C) gave identical results, indicating that micellar solubilization is not essential. Third, at any given temperature the final droplet composition should be given by the equilibrium liquidus line (Fig. 1). However, provided that the *p*-CNB does not crystallize within the drops, there is not reason why *m*-CNB crystals should not continue to grow as a pure phase, with the drops becoming smaller and further enriched with *p*-CNB. Such a

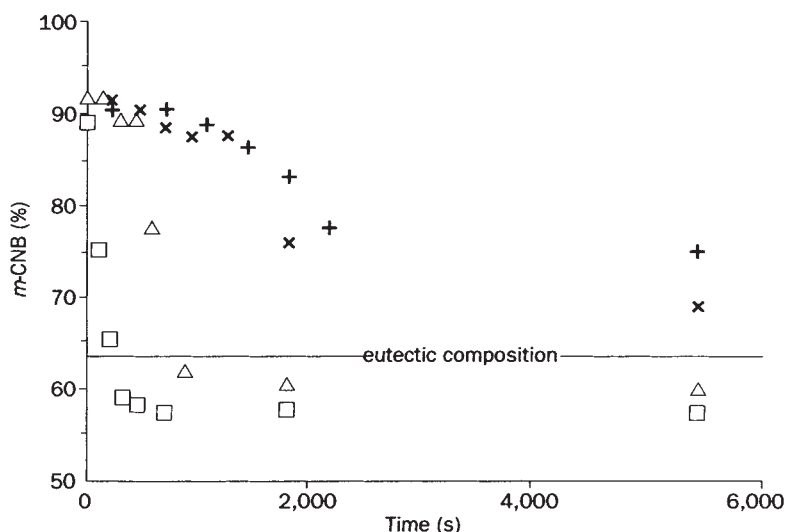


FIG. 3 Time dependence of emulsion droplet composition at different temperatures (+, 30 °C; x, 28 °C; Δ, 24 °C; □, 22 °C). Total crystallization time, 90 min.

feature of this system is entirely consistent with purification below the eutectic; homogeneous nucleation of *p*-CNB in the drops will be inhibited by their decreasing size, and continued transfer of *m*-CNB from drops to crystals will take the liquid phase through the eutectic into the *p*-CNB side of the phase diagram.

To test this mechanism, emulsions having a *m/p* composition 90:10 were crystallized at temperatures of 30, 28, 24 and 22 °C and the emulsion droplet composition measured as a function of time (Fig. 3). At 24 and 22 °C the liquid phase does not have the eutectic composition (62.9%), but rather a composition richer in *p*-CNB, with respective crystal purities (Table 1) of 98.2 and 93.6% *m*-CNB.

Finally we took an emulsion of composition 60.9% *m*-CNB and crystallized it (with *m*-CNB seeds) at 15 °C, where we would expect a mixture of pure *p*-CNB and eutectic to crystallize. Contrary to this expectation, we obtained a final crystal purity of 65.5% *m*-CNB and a final liquid phase composition of 55.3% *m*-CNB. Thus enrichment of *m*-CNB was achieved on the *p*-CNB

side of the eutectic, confirming that bypassing the eutectic is a real possibility in this emulsion system.

It is our belief that including a specific crystallization inhibitor for the unwanted component of the mixture (*p*-CNB here) would offer further enhancement of the separation by stabilizing the emulsion against crystallization at still lower temperatures. □

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Interannual extremes in the rate of rise of atmospheric carbon dioxide since 1980

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OBSERVATIONS of atmospheric CO₂ concentrations at Mauna Loa, Hawaii, and at the South Pole over the past four decades show an approximate proportionality between the rising atmospheric concentrations and industrial CO₂ emissions¹. This proportionality, which is most apparent during the first 20 years of the records, was disturbed in the 1980s by a disproportionately high rate of rise of atmospheric CO₂, followed after 1988 by a pronounced slowing down of the growth rate. To probe the causes of these changes, we examine here the changes expected from the variations in the rates of industrial CO₂ emissions over this time², and also from influences of climate such as El Niño events. We use the ¹³C/¹²C ratio of atmospheric CO₂ to distinguish the effects of interannual variations in biospheric and oceanic sources and sinks of carbon. We propose that the recent disproportionate rise and fall in CO₂ growth rate were caused mainly by interannual variations in global air temperature (which altered both the terrestrial biospheric and the oceanic carbon sinks), and possibly also by precipitation. We suggest that the anomalous climate-induced rise in CO₂ was partially masked by a slowing down in the growth rate of fossil-fuel combustion, and that the latter then exaggerated the subsequent climate-induced fall.

An unexpected slowing in the rate of rise of atmospheric CO₂ appeared recently in measurements of CO₂ made at Mauna Loa Observatory, Hawaii and the South Pole (Fig. 1a). To study this and other interannual changes, both short-term and decadal, we have seasonally adjusted both records and then averaged them (Fig. 1b). The solid curve in the same panel depicts cumulative industrial emissions of CO₂ (mainly from the combustion of fossil fuels³) and shows the near proportionality of these emissions with the observed atmospheric CO₂ concentration increase. The fraction of cumulative emissions has been set to agree with the observed increase in atmospheric CO₂ between 1959 and

1979, a 20-year baseline period when industrial emissions grew at a nearly constant rate of 4.0% per year, leading to the expectation that a nearly constant fraction of these emissions should remain airborne (see p. 89 of ref. 3). By subtracting this curve of industrial emissions from the CO₂ record in Fig. 1b, disproportionate variations become clearly discernable (Fig. 1c).

To investigate the causes of these disproportionate variations, we first consider the expected influence of changes in the growth rate of industrial CO₂ emissions. To compute this influence, we have used a carbon-cycle model¹ which predicts the expected direct responses of the global terrestrial-biospheric and oceanic carbon reservoirs to industrial CO₂ emissions. The model accounts for changes in reservoir response directly related to the build-up of atmospheric CO₂, but not for the influence of environmental factors related to climatic variability.

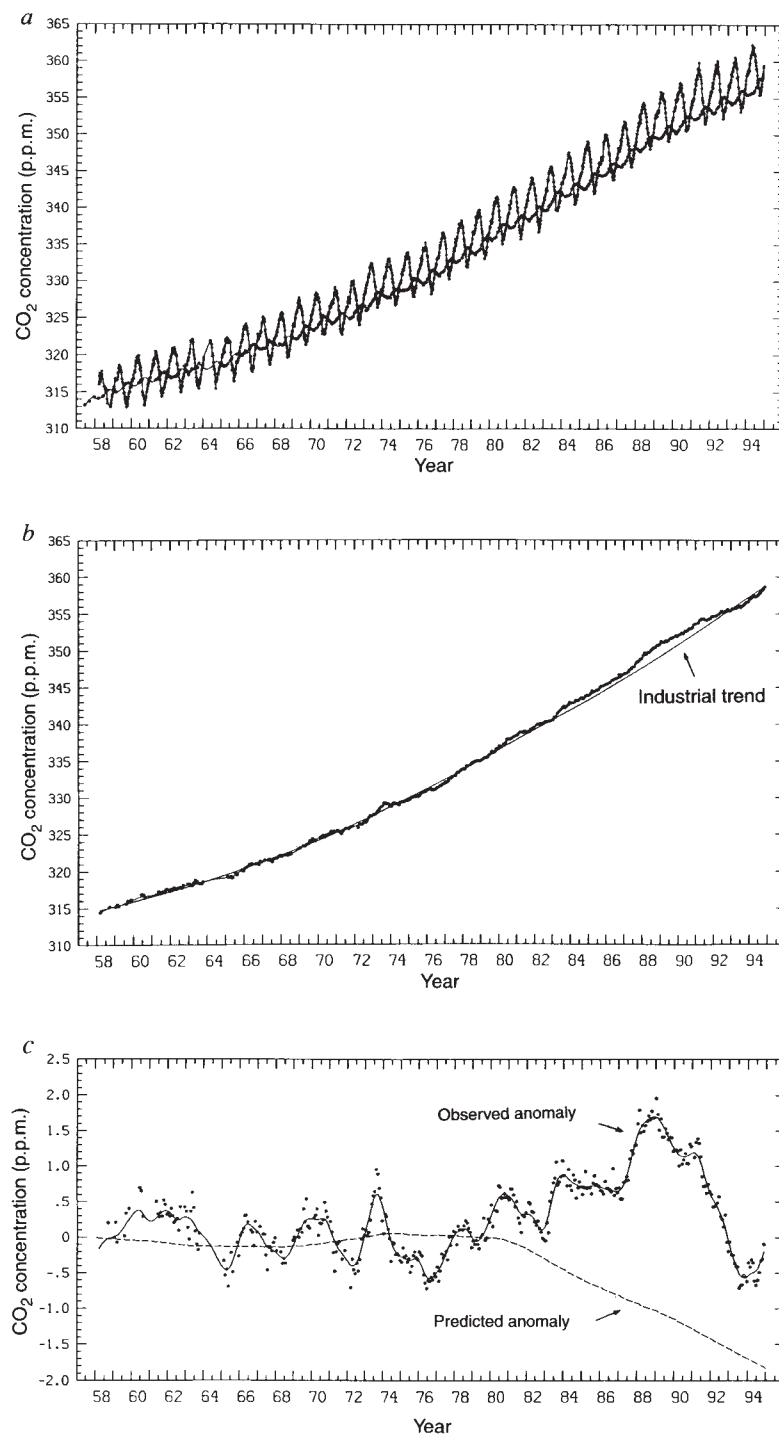
With the model adjusted so that the observed fraction of industrial emissions over the 20-year baseline period remains airborne (55.9%), and provided that observed short-term fluctuations are disregarded, the predicted anomaly (Fig. 1c, dashed curve) fulfils the expectation of a constant airborne-fraction during the baseline period by having remained near zero, as did the observed anomaly. After 1980, however, the predicted anomaly fell steadily in response to an abrupt decrease in the growth rate of industrial emissions to an average of 1.7% (discernible in Fig. 1b as a decrease in upward curvature of the industrial trend after 1979). Changes after 1980 in the reported annual growth rate of industrial emissions² (data available to the end of 1991; 1% growth rate assumed for 1992 and 1993) are seen in Fig. 1c to have had little effect on the steadiness of the predicted downward trend. At the same time, the observed anomaly tends to rise, and, in spite of a steep decline after 1991, has remained above the model-predicted anomaly ever since.

The marked discrepancy between the predicted and observed anomalies on the decadal timescale after 1980, and differences throughout the record on shorter timescales, may be related to climate forcing involving air temperature⁴, because anomalous variations in CO₂ and in temperature have tended to occur coherently, as suggested by comparing Fig. 2a with Fig. 2b. Many of these coherent anomalies are associated with El Niño events (arrows in Fig. 2a), but they also occur on the decadal timescale (solid curves) as previously noted by Keeling *et al.* (see p. 211 of ref. 1), and confirmed by a rigorous analysis of coherence⁴. On the decadal timescale a negative correlation of CO₂ with globally averaged precipitation over land is also suggested (Fig. 2c, note inverted plot).

Fluctuations in CO_2 associated with El Niño events have lagged those in temperature, as expected (see p. 211 of ref. 1) if the rate of change in CO_2 concentration were directly influenced by the temperature. In contrast, the decadal variations in temperature, and possibly in precipitation, almost directly correlate with the CO_2 concentration itself. If these decadal correlations are significant, it seems evident that the onset of a climate change, such as a warming trend, has a measurable influence on the atmospheric CO_2 concentration, but that if the climate change is sustained, its further influence gradually weakens. Possible reasons for this weakening influence may involve time delays between coupled processes of uptake and release of carbon, for example between perturbations in photosynthesis and respiration of plant communities.

To add further to our understanding of the possible causes of these anomalous changes in CO_2 , we have compared variations in the concentration of atmospheric CO_2 with variations in the $^{13}\text{C}/^{12}\text{C}$ isotopic ratio of this gas in air collected at both Mauna Loa Observatory and the South Pole (Fig. 3a and b). By taking into account isotopic fractionation of carbon in the major pathways of the global carbon cycle, we have attempted to distinguish the contributions to the atmospheric CO_2 anomaly caused separately by gas exchanges with the terrestrial biosphere and with the oceans¹⁻⁵. This calculation, which has been called a "double deconvolution"¹, is not without uncertainty, because the isotopic fractionation factors and ratios involved in the pathways and reservoirs of the carbon cycle are not accurately known, and the signal associated with El Niño events is relatively

FIG 1 Time trend in the concentration of atmospheric CO_2 , in parts per million by volume (p.p.m.), compared to emissions of CO_2 from fossil-fuel combustion. *a*, The concentrations of CO_2 at Mauna Loa Observatory, Hawaii (20°N , 156°W) and at the South Pole through November 1994. The former record is distinguished by its pronounced seasonal cycle. For Mauna Loa, weekly data are shown as dots and connected with straight lines. For the South Pole, monthly data points are shown as dots, and connected approximately by a smooth curve (see p. 167 of ref. 1). *b*, The average of the above records (dots connected by a smooth curve as in *a* for the South Pole) after seasonal adjustment of monthly averages at both stations, compared to a curve (solid line, labelled 'industrial trend') of a fixed fraction (55.9%) of the cumulative industrial emissions of CO_2 from fossil fuel combustion and cement production². This fraction was chosen to bring exact agreement between the observations and cumulative industrial emissions between 1 January 1959 and 1 January 1979, and is almost the same fraction as realized over the entire record. *c*, Observed CO_2 anomaly obtained by subtracting the industrial emissions curve in *b* from the monthly CO_2 data. The solid curve is a spline fit^{1,17} to the monthly data with a standard error, σ , of 0.096 p.p.m., selected to emphasize the fluctuating character of the record on the timescale of El Niño events (large-scale, coherent climate anomalies associated with a collapse of the trade winds over the Pacific ocean^{18,19}). The dashed curve depicts a predicted CO_2 anomaly, in which it is assumed that variability in atmospheric CO_2 is caused solely by industrial CO_2 emissions modified by the direct responses of the global terrestrial biospheric and the oceanic carbon reservoirs to these emissions, neglecting any effect of climate factors. The prediction is based on a carbon-cycle model¹, in which the oceanic carbon reservoir is assumed to transport carbon by vertical diffusion at a rate that reproduces the observed uptake of radiocarbon, and the terrestrial biosphere is assumed to respond to higher CO_2 concentrations by more rapid plant growth. A global growth factor was set to 0.114 so that a constant fraction (55.9%) of industrial CO_2 emissions is predicted to remain in the atmosphere between 1959 and 1979.



weak. Nevertheless, the results of double deconvolution suggest an explanation for the observed anomalies in CO₂ concentration which we believe to be qualitatively correct.

As shown in Fig. 3c and d, the inferred separate biospheric and oceanic fluxes contributing to the CO₂ anomaly, since 1978 when our isotopic record began, have tended to oppose each other, with increases in biospheric sources and anomalous oceanic sinks occurring at times of rising CO₂ anomaly (Fig. 2a) and the reverse occurring at times of falling CO₂ anomaly¹. The period of sharply falling anomaly after mid-year 1991 is an exception, in which for slightly over a year, sinks are indicated for both fluxes⁶, perhaps a rare event in light of our analysis of El Niño events.

Disregarding short-term interannual fluctuations, the anomalous oceanic flux, in the 12.5 years of isotopic record before June 1991, was a source of 3.0 Gt C, consistent with significant warming of surface water which occurred during this period (see p. 204 of ref. 1). The overall negative biospheric flux of 1.2 Gt C, for the same period only partially offsets this oceanic source. In contrast, for approximately the next two years (June 1991 to September 1993) the anomalous oceanic flux was negligible while the biospheric flux represented a strong sink of 3.0 Gt C.

Taking note of the short-term fluctuations in global CO₂ anomaly plotted in Fig. 2a, one sees successively higher minima in 1979, 1982, 1986 and 1990 in advance of El Niño events. After the minimum in 1990, the rise was brief, however, being cut short in mid-year 1991 by a steep decline. Is the Pinatubo volcanic eruption⁷ of June 1991 implicated in this dramatic down-

ward anomaly? A search for a connection between this event and the carbon cycle is complicated by weak El Niño conditions which were present during both 1992 and 1993 in the tropical Pacific⁸. Our double-deconvolution calculation suggests that the oceans typically are a larger sink for atmospheric CO₂ during El Niño events than otherwise^{1,9}, whereas the terrestrial biosphere is the reverse. The inferred anomalous oceanic sink of 0.6 Gt C immediately after the Pinatubo eruption (Fig. 3d), although weak and soon cancelled by a weak source in 1993, is consistent with this explanation. The biosphere, however, unlike other recent El Niño events, was also a sink as noted above.

Cooling produced by volcanic aerosols from the eruption might explain this unusual biospheric sink, especially if the sink preceding it in 1989 and 1990 was mainly a recovery from a biospheric source during the El Niño event that began in 1987. The magnitude of the post-1991 cooling trend, shown in Fig. 2b, although less precipitous than the fall in the CO₂ anomaly, was greatest during the northern summer months (see short-dashed portion of curve in Fig. 2b). This cooling, if it was accompanied by increased precipitation at appropriate locations in the Northern Hemisphere, may have appreciably enhanced plant growth (see p. 167 of ref. 10) or reduced the release of CO₂ by soil respiration and wildfires.

Perhaps, however, the Pinatubo eruption was only incidental to a declining CO₂ anomaly. The unusually warm temperatures preceding the eruption may have caused an increase in photosynthesis and hence in net biospheric uptake of CO₂ over the whole period of falling CO₂ anomaly after 1988. A substantial

FIG 2 Comparison of the interannual variability of anomalies in atmospheric CO₂, surface air temperature and atmospheric precipitation. a, Monthly CO₂ anomalies as shown previously in Fig. 1c except that the previously solid curve is now shown as a dashed curve, and the data extend only through May 1994. The new solid curve represents a spline fit ($\sigma = 0.129$ p.p.m.) to emphasize decadal timescale variability. Vertical arrows indicate times of El Niño events^{1,8,20}. A vertical line, also shown in b, indicates the time of the main eruption of Mt Pinatubo⁷. b, A similar plot of global land and oceanic near-surface air temperature variability (σ is 0.121 and 0.230 °C for dashed and solid curves, respectively). The monthly data, to and including 1993, are from refs 21–24 and P. D. Jones, personal communication). c, Plot of percentage deviations from the mean in global precipitation over land to 1989, reproduced from Fig. 17 of Eischeid *et al.*²⁵ inverted to emphasize a negative correlation of precipitation with CO₂ on the decadal timescale. The dashed line connects annual averages shown as dots. The solid line, from the original figure, is a smoothed representation to indicate long-timescale variations.

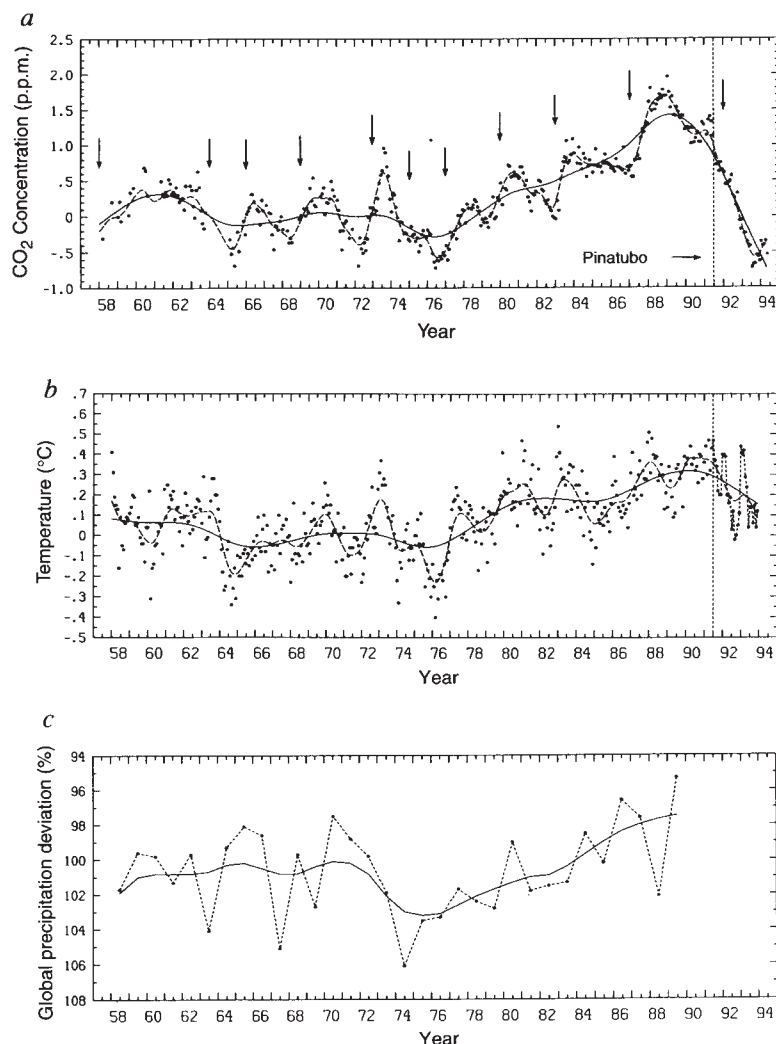
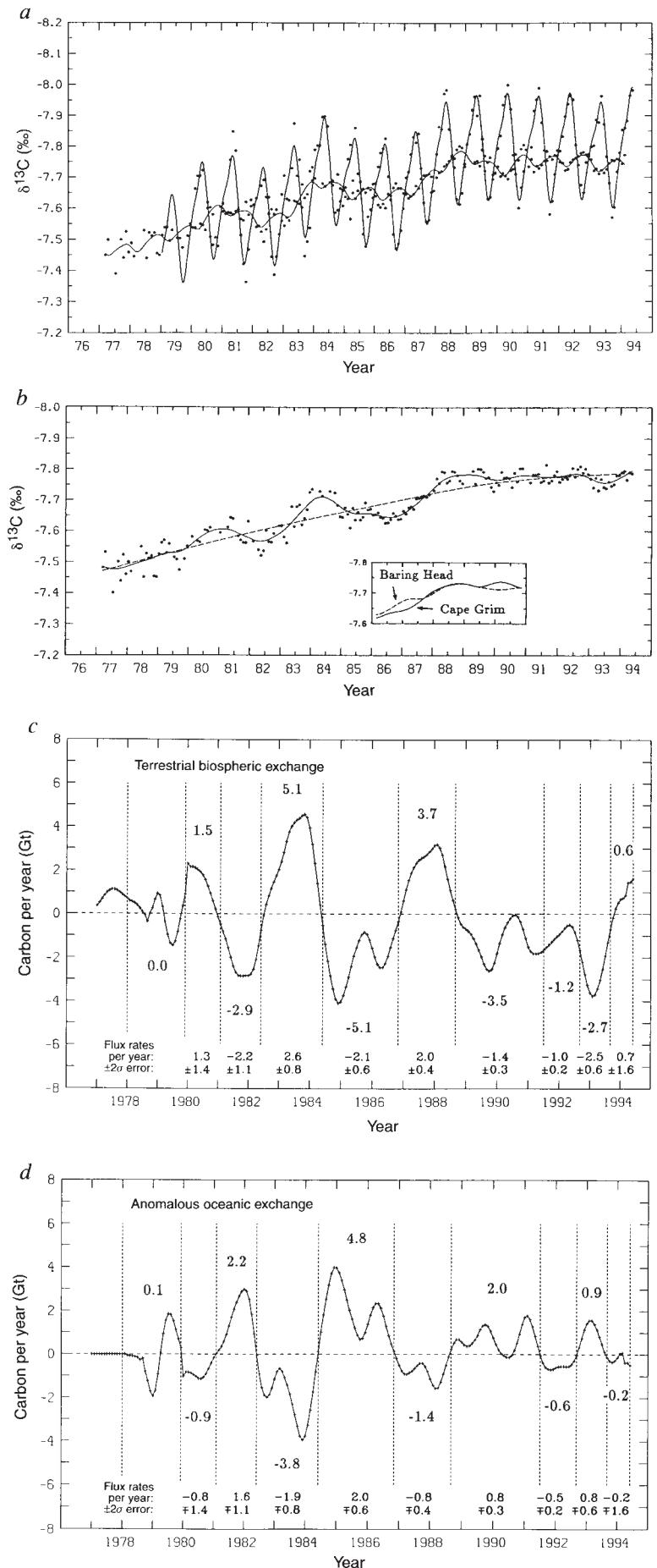


FIG 3 Time trend in the reduced isotopic ratio, $\delta^{13}\text{C}$, of atmospheric CO_2 from 1977 through early 1994, shown together with time plots of interannual exchanges of atmospheric CO_2 by the oceans and terrestrial biosphere. The exchanges are inferred from these isotopic data in combination with the atmospheric CO_2 data shown in Fig. 1a. a, The reduced isotopic ratio, $\delta^{13}\text{C}$ at Mauna Loa Observatory and the South Pole, the former record distinguished by its pronounced seasonal cycle (compare Fig. 1a). The ratio $\delta^{13}\text{C}$ is defined as $(R/R_0) - 1$, where R denotes the $^{13}\text{C}/^{12}\text{C}$ ratio of the sampled gas and R_0 is the corresponding ratio of the international carbonate standard, PDB. The data, in per mil (‰), are shown as monthly averages by dots and the trends by solid curves (compare Fig. 1a). b, The average of the above records after seasonal adjustment (compare Fig. 1b). The data have a standard error, σ , of 0.024 (‰) relative to the solid curve, which follows the interannual trend as closely as possible, and a σ of 0.037 (‰) relative to the stiffer dashed curve, in which shorter-term interannual variations are suppressed by decreasing the squared second derivative of the fitting function²⁶. Inset, similar trends at Baring Head, New Zealand (41° S, 175° E) from data measured in our laboratory (dashed line) and at Cape Grim, Tasmania (41° S, 145° E) as reported by Francey *et al.*¹⁶ (solid line). These trends show a lesser-amplitude El Niño signal, as discussed in the text. The σ values of these curves (0.042 and 0.029 ‰, respectively) were set using data from 1985 to 1991 to give the same squared second derivative²⁶, so that the stiffnesses are the same. c, Time trend in the exchange of CO_2 by the terrestrial biosphere with the atmosphere, determined by a double deconvolution and shown as a solid curve, in Gt C yr^{-1} (where Gt C denotes 10^{12} kg of carbon). The calculation of CO_2 solubility in sea water assumes constant temperature (also in Fig. 3d). The exchanges are not adjusted to sum to zero over the full record (as in ref. 1, Figs 55 and 56) and thus their averages over the full record reflect the oceanic vertical diffusion rate assumed in the double deconvolution¹. Vertical lines separate periods of persistent positive and negative fluxes whose integrated fluxes are shown numerically in Gt C , directly above or below the curve. They are defined by the zero crossings in d. Flux rates are shown at the bottom of the panel in Gt C yr^{-1} with estimates of their statistical error expressed as $\pm 2\sigma$ (95% confidence level). d, Residual (anomalous) exchange of CO_2 by the oceans, determined by the double deconvolution after allowing for oceanic absorption of CO_2 predicted in response to industrial CO_2 emissions and the isotopic disequilibrium attending that response. The periods of integration, shown by vertical lines, define alternations between positive and negative fluxes that approximate warm and cold phases of El Niño events. The statistical error analysis was performed by carrying out a second double deconvolution, assuming the stiffer dashed curve of Fig. 3b in place of the solid curve. The difference in Gt C over each interval of integration, found by comparing the two double deconvolutions, was taken as the basis for scaling the statistical error in flux to the error in the trend in $\delta^{13}\text{C}$, the latter being approximated by a straight-line fit to the data of the interval. Because the flux is proportional to the slope, the error in flux is proportional to the error in the slope. Computed errors in $\delta^{13}\text{C}$ contribute equally and oppositely to the biospheric and oceanic fluxes, because these sum to the flux obtained by the better-constrained single deconvolution calculation (see p. 202, equation 6.6 of ref. 1) based only on concentration data which we assume to contribute no error. This statistical analysis does not address unidentified systematic biases in the data.



increase in the amplitude of the seasonal oscillation of atmospheric CO₂ from 1989 through 1993 (we calculate an 8% increase at Mauna Loa, Hawaii, compare Fig. 1a) suggests this possibility. Or perhaps cooling related to the Pinatubo eruption and antecedent warming both contributed to a biospheric sink after 1989.

Changes in human land use, especially deforestation, have added further emissions of CO₂ to the air in recent years, and may partially account for the rising CO₂ anomaly after 1979. This is difficult to prove, however, because little information exists to establish annual rates of CO₂ emissions from land-use change¹¹. Even the global average emission rate since 1979 is poorly known, as indicated by a wide range of estimates¹² (0.4–2.6 Gt C yr⁻¹). It is not likely, however, that a substantial part of the shift from an upward to a downward-tending anomaly after 1988 was due to land-use changes. The decrease in CO₂ emission rate required to explain the shift is too large, being approximately 1.4 Gt C yr⁻¹ after taking into account that oceanic CO₂ exchange must partially offset biospheric exchange, as it does with respect to industrial emissions. Some decrease in deforestation may have occurred about this time^{13,14}, but almost surely not a decrease approaching 1.4 Gt C yr⁻¹, a change greater than half of the upper limit of the global emission rate from land-use change, quoted above. Therefore, the shift is more likely to reflect mainly natural causes, most evident in the correlation of CO₂ anomalies with temperature, seen in Fig. 2a and b.

In summary, the slowing down of the rate of rise of atmospheric CO₂ from 1989 to 1993, seen in our data and confirmed by other measurements^{6,15}, is partially explained (about 30% according to Fig. 1c) by the reduction in growth rate of industrial CO₂ emissions that occurred after 1979. We further propose that warming of surface water in advance of this slowdown caused an anomalous rise in atmospheric CO₂, accentuating the subsequent slowdown, while the terrestrial biosphere, perhaps by sequestering carbon in a delayed response to the same warming, caused most of the slowdown itself.

In contradiction to some of our findings, Francey *et al.*¹⁶ have recently suggested that isotopic data for atmospheric CO₂ from Cape Grim, Tasmania (41° S) and elsewhere do not show an El Niño signal. For the 1983 El Niño event their data in this respect clearly disagree with our data at Mauna Loa and the South Pole¹, but the comparison is not rigorous because we lack data for the vicinity of Cape Grim. For the 1987 event, however, we have data for Baring Head, New Zealand, a station at nearly the same latitude as Cape Grim, and they have data for the South Pole¹⁶. Our New Zealand data show an El Niño signal that is a little more than half as strong as we find for the South Pole and Mauna Loa averaged. This weaker signal agrees well with a signal which we find in their Cape Grim data after seasonal adjustment (Fig. 3b inset), a signal which, however, was reduced in their analysis by smoothing (their Fig. 1a).

Furthermore, their data for the South Pole show a signal similar to ours for the 1987 event, although they did not include these data in the computation of sources and sinks of CO₂. On the basis of the near agreement of our and their data sets at both latitudes, it seems plausible that El Niño isotopic signals are generally weaker in the middle of the Southern Hemisphere than elsewhere, perhaps a consequence of isotopic air–sea exchange. (We find no corresponding diminution in the concentration signal in the middle of the Southern Hemisphere.) Thus, Francey *et al.* may have reached a conclusion contrary to ours by focusing on their isotopic data for a single station (Cape Grim) where the El Niño signal may be weaker. Thus, we believe that our isotopic data are correct in inferring opposing global oceanic and terrestrial El Niño CO₂ signals, although these may have been exaggerated by our not taking into account isotopic data in the middle of the Southern Hemisphere.

We point out, in closing, that the unprecedented steep decline in the atmospheric CO₂ anomaly ended late in 1993 (see Fig.

1c). Neither the onset nor the termination was predictable. Environmental factors appear to have imposed larger changes on the rate of rise of atmospheric CO₂ than did changes in fossil fuel combustion rates, suggesting uncertainty in projecting future increases in atmospheric CO₂ solely on the basis of anticipated rates of industrial activity. □

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Archaean subduction inferred from seismic images of a mantle suture in the Superior Province

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PLATE tectonics provides the basis for the interpretation of most current terrestrial tectonic activity, and is widely accepted as having been active over much of the Earth's history¹. Yet the timing of initiation of this process is subject to debate^{2–9}. So far, the earliest seismic evidence for plate tectonics has come from a fossil mantle suture in the Svecofennian orogen (1.89 Gyr ago)¹⁰ and

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from inferred plate convergence, subduction and accretion in the Trans-Hudson orogen (1.91–1.79 Gyr ago)¹¹. As yet, seismic data from Archaean areas have been able to demonstrate only the importance of compression in the construction of the continental crust^{12–15}. Here we present seismic data from a collision zone in the Superior Province of Canada, involving the Abitibi granite-greenstone Subprovince and the plutonic, arc-related Opatica belt. We interpret dipping seismic reflections that extend 30 km into the mantle as representing a relict 2.69-Gyr-old suture associated with subduction. Although crustal structure, lithospheric thicknesses and convergence rates may have differed from those seen today, these seismic data provide direct evidence that plate tectonics was active in late Archaean times, 800 Myr earlier than indicated by previous seismic reflection surveys.

The Superior Province of Canada consists of an alternating sequence of granite-greenstone belts and metasedimentary subprovinces that decrease in age from 3,100 Myr in the north to 2,650 Myr in the south (Fig. 1). This sequence probably arose through the amalgamation of progressively younger terranes along arcuate, primarily north-facing subduction zones¹⁶. The world's largest greenstone belt, the Abitibi Subprovince, lies in the southeastern corner of the Superior Province; it is less than 2,730 Myr old, and is dominated by juvenile magmatism¹⁷. The most northerly region of the Abitibi belt, the northern volcanic zone (NVZ), comprises subaqueous basaltic sequences and occasional felsic volcanic centres together with their associated sedimentary deposits. The NVZ was deformed and metamorphosed to greenschist facies during a north-south compressional event between 2,708 and 2,694 Myr ago^{18,19}. Subsequently, a system of approximately east-west trending right-lateral strike-slip faults developed^{15,18}. The northern boundary of the Abitibi Subprovince is defined by the contact of the NVZ with the high-grade tonalitic gneiss of the Opatica belt²⁰.

The Opatica belt formed over a period of ~150 Myr, from pre-2,825 Myr to 2,680 Myr, and contains plutonic rocks that are significantly older than the Abitibi belt to the south²¹. The tonalitic gneiss and granitoid rocks of the Opatica belt are highly deformed with moderately dipping fabrics that contrast sharply with the subvertical structures mapped to the south in the Abitibi belt. Pervasive, crustal-scale WSW-vergent high-temperature, ductile shearing occurred between 2,702 Myr and 2,693 Myr ago, creating a well developed gneissic foliation. Subsequently, a SSE-vergent thrusting event imbricated the Opatica belt forming a large antiform in the south^{22,23}. A northward increase in metamorphic grade, to upper amphibolite facies, suggests that the Opatica belt is the deeply eroded core of an Archaean orogen,

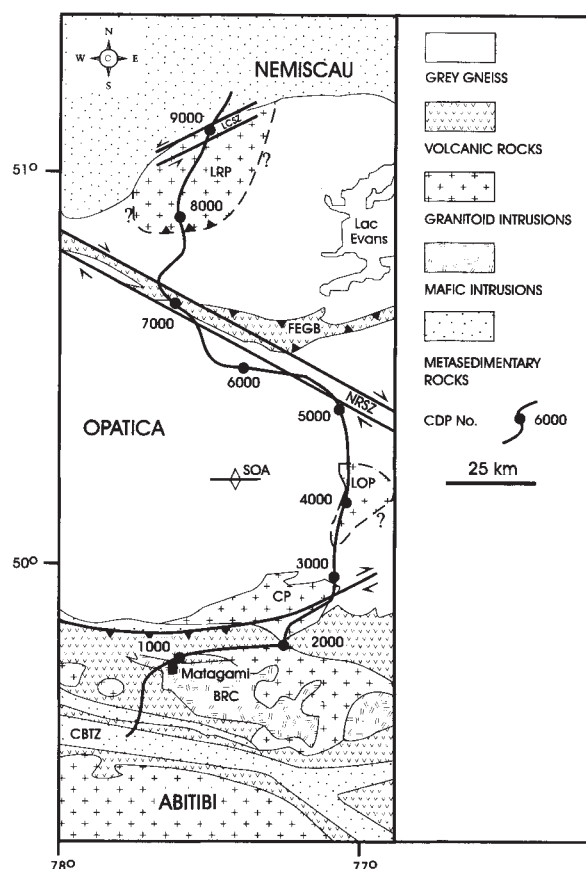


FIG. 2 Location of seismic reflection profile 48 extending from the Casa Berardi tectonic zone (CBTZ) in northern Abitibi across the Opatica belt and terminating in the Nemiscau Subprovince, considered to be part of the Opatica shown in Fig. 1. BRC, Bell River complex; CP, Canet pluton; LOP, Lac Ouescapis pluton; LRP, Lac Rodayer pluton; SOA, axis of the eroded southern Opatica antiform; NRSZ, Nottaway River shear zone; FEGB, Frotet-Evans greenstone belt; LCSZ, Lac Coulomb shear zone.

possibly associated with a southward-propagating foreland fold and thrust belt in which gneisses were thrust beneath the NVZ^{22,24}. The Opatica belt is cut by a number of approximately southeast-trending subvertical shear zones. The most notable of these is the Nottaway River shear zone (NRSZ), which traverses the entire belt and may extend into the adjacent subprovinces, thus postdating their amalgamation.

Seismic line 48 was shot as part of the third phase of seismic reflection acquisition for the LITHOPROBE Abitibi-Grenville transect. The survey objectives were to define the contact at depth of the Abitibi belt with the Opatica Subprovince and to determine the validity of the Opatica orogenic model. The seismic line extends across the entire Opatica belt crossing three major plutons, the NRSZ and the small Frotet-Evans greenstone belt (FEGB), which lies entirely within the NRSZ at this location (Fig. 2). Figure 3 shows a section of line 48 from the centre of the Opatica belt. It clearly indicates seismic reflections at all crustal levels. The upper mantle appears largely transparent with the exception of a band of dipping reflections between 17 and 20 s. The reflective lower crust, well-defined Moho and dipping mantle reflections of the Opatica belt, are unique to date amongst seismic observations from Archaean areas. Both stack and migrated sections of line 48 were used to define the structural relationships and reflector orientations, with special care being taken where the direction of the seismic line changed.

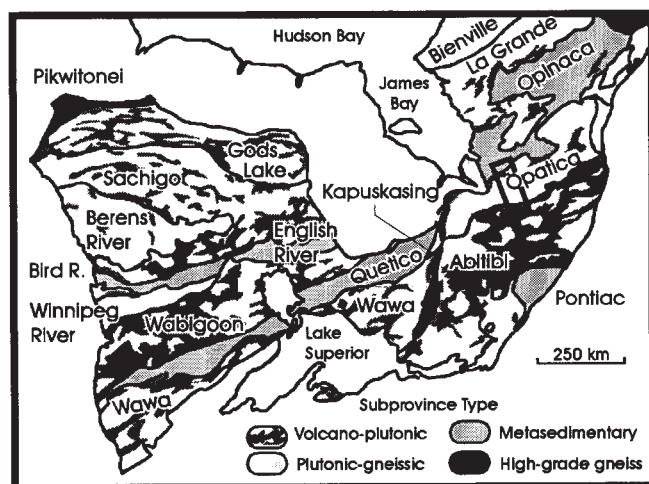


FIG. 1 Geological map of the Superior Province showing the alternating metasedimentary and granite-greenstone belts. The study area over the plutonic Opatica belt is indicated.

The greenstone rocks of the Abitibi upper crust, with the exception of the 45°-dipping Bell River mafic intrusion near common depth point (CDP) 600 of line 48, are comparatively unreflective (Fig. 4). The base of the greenstones is interpreted to be the reflection zone that shallows slightly from 3.2 s at CDP 100 to 2.7 s at CDP 1,900, because it marks the transition to the stronger reflectivity of the middle crust and is not cut by steeply dipping overlying reflections. The subhorizontal reflections may represent a thrust contact consistent with emplacement of this part of the NVZ onto Opatoca orthogneiss as an allochthonous thrust sheet²². We thus interpret the thickness of the greenstone rocks to be ~10 km, in agreement with the results of other surveys in this area²⁵. Steep, north-dipping reflections at the south end of line 48, which rise to 3 s, truncate mid-crustal reflections to the north, probably marking the southern limit of Opatoca crust.

A thin set of shallowly north-dipping reflections (D in Fig. 4) extend from CDP 1 at 7.0 s to the base of the crust at 12 s near CDP 5,000 and marks a change in reflection character from

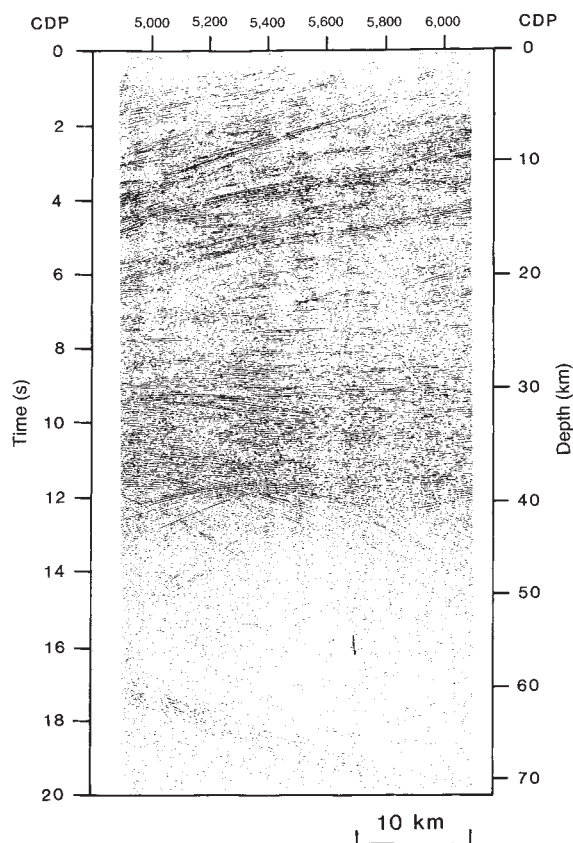


FIG. 3 Part of seismic reflection profile 48 showing reflections from the mantle between 17 and 20 s. The crust-mantle boundary is well defined at 12 s by the sharp termination of the subhorizontal lower-crustal reflectivity. A number of diffractions, which extend to 14.0 s in this unmigrated display, originate close to this contact. The seismic line was recorded with a 400-channel telemetry acquisition system using 34 m long linear arrays of nine geophones deployed every 50 m. Four vibrators, each with a peak force of 22,800 kgf (50,160 lbf) were deployed in a seven-sweep, 100-m source array; the vibration point spacing was 100 m. A linear, 14-s 10–56 Hz upsweep was employed, and the data were recorded with a 60-Hz notch filter to facilitate diversity stacking in the presence of electrical power-line noise. A conventional processing sequence was applied to the data: refraction statics, crooked line binning, minimum phase conversion, dynamic equalization, spiking deconvolution, velocity analysis, normal moveout, mute, surface consistent residual statics and stack. No poststack processing or gain has been applied to the stack data in this display.

middle to lower crust. We interpret this boundary as a lower-crustal decollement, as overlying, steeply dipping mid-crustal reflections become flatter and merge into it. North of CDP 3,000, the decollement cuts through Opatoca lower-crustal reflectivity, suggesting that lower crust originally associated with the Opatoca unit underlying the Abitibi greenstones may have been displaced northwards and tectonically imbricated by thrusting beneath the decollement. Two sets of reflections with opposing dips that extend close to the decollement are observed within the upper and middle crust of the Opatoca belt between CDP 2,500 and 6,000 (Fig. 4b).

They are approximately symmetric about the axis of the eroded southern Opatoca antiform (SOA). Near CDP 5,000 these upper and mid-crustal reflections are estimated to dip to the SSE. This correlates well with the orientation of a number of mylonitic shear zones and backthrusts mapped at the surface²², suggesting that much of the high-amplitude reflectivity is associated with these shear zones along which mid-crustal rocks were exhumed. Mantle reflections intersect the Moho just to the south of SOA, and dip north to northwest. We infer that both the mantle reflections and the two sets of oppositely vergent shear zones were created in the same tectonic event, the SSE-vergent thrusting, because they have parallel strike directions. Such a link is also suggested, in general, by geodynamical modelling studies which show that two oppositely vergent belts of deformation (the pro and retro regions), initially rooted at a decollement, develop above the location of subduction into the mantle^{26,27}.

Strong lower-crustal reflections, some continuous over 20 km, are observed beneath the Opatoca belt. The reflection Moho is also well defined, undulating slightly between 11.5 and 12.0 s and implying a crustal thickness of 38–40 km. Archaean lower crust has often been characterized as unreflective^{12,14,28} and underlain by a gradual transition from crustal to mantle rocks^{29,30}. Our results suggest, in contrast, that the reflection Moho can be a very sharp boundary and that the style of reflective lower crust often observed in Phanerozoic and Proterozoic areas does occur in Archaean crust. We interpret the sharp Moho to be a shear zone created in response to the shortening and imbrication of the lower crust above a more rigid upper mantle. Poorly defined Mohos in other Archaean areas could be attributable either to the absence of such deformation or to a subsequent, possibly thermal, modification of the lower crust that destroyed most of any existing reflectivity. At the south end of line 48 the crust-mantle transition is less clear, but an enigmatic 'step' in the Moho is observed at CDP 1,300, where the time to the deepest lower-crustal reflections decreases from 13.5 to 11.0 s. The change in orientation of the seismic line does cause the amplitude of some reflections to vary laterally, but this cannot explain the termination of reflectivity at 11.0 s, which is clearly observed between CDPs 1,700 and 2,500. Furthermore, when the mantle reflections are followed up to 11.5 s their dip decreases to become almost subhorizontal before the seismic line changes direction at CDP 2,650 (Fig. 4). We thus tentatively infer the presence of a unit below the mantle reflections that continues south to the 'step' in the Moho.

The mantle reflections extend to at least 65 km depth. Mantle reflections have been reported beneath Phanerozoic and Proterozoic crust and attributed to shear zones associated with both extension and compression^{10,31,32}. Detailed analysis of mantle reflections off northwest Scotland suggests that they may originate at the upper surface of an eclogitic slab embedded in the mantle by subduction³³. Our preliminary interpretation of the mantle reflections observed on line 48 is that they arise from a shear zone created by thrusting of a lower plate down into the mantle, and thus represent an upper-mantle suture. The proposed link with the overlying crustal reflectivity implies that the suture was created in the SSE-vergent thrusting event mapped at the surface and dated at ~2,690 Myr ago. As Archaean upper-mantle temperatures were perhaps 200–300 °C higher than present-day values^{34,35}, loss of volatiles in the subducted slab

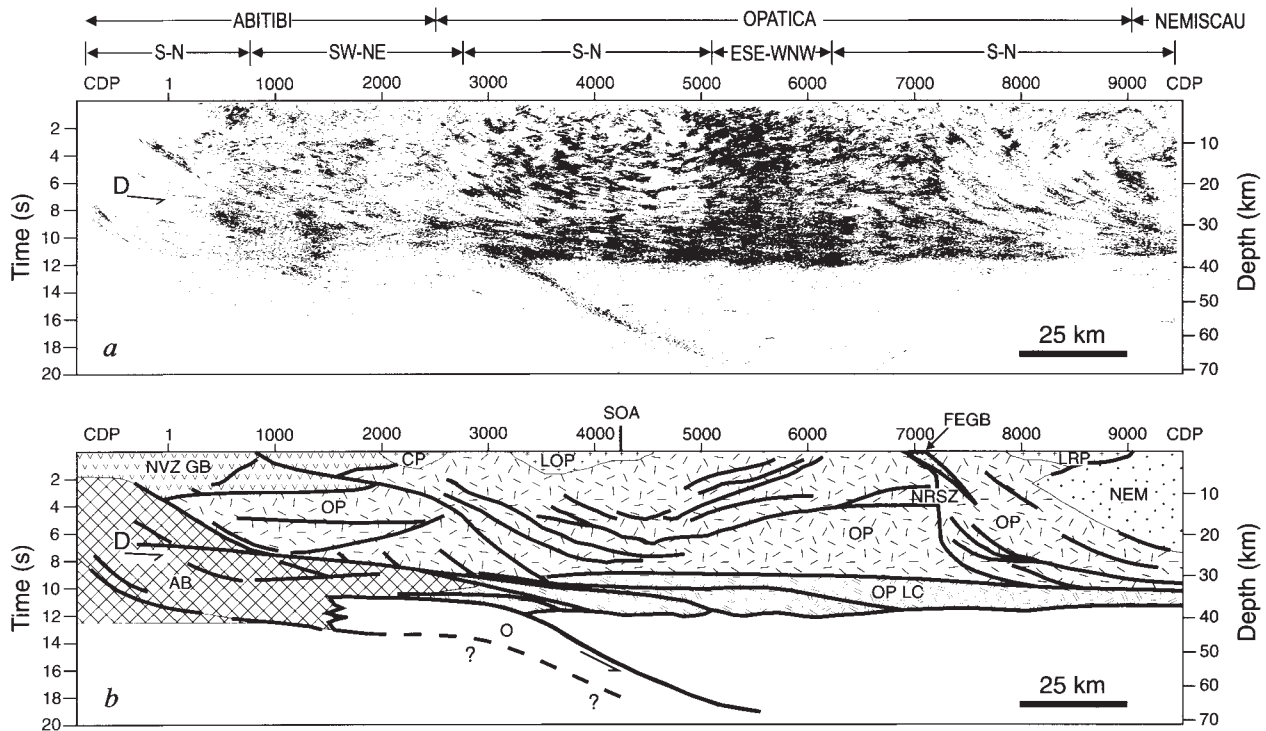


FIG. 4 *a*, Line migration of line 48 displayed at true scale (1:1) derived by migrating the stack at $6,500 \text{ m s}^{-1}$ using apparent local dips estimated over a 21-trace window; this method avoids artefacts associated with wave-equation migration and provides a better image in the mantle. The most prominent feature of the data is the band of mantle reflections that dip in a north to northwest direction beneath the Opatica belt. The mantle reflections intersect the Moho beneath the Abitibi–Opatica boundary mapped at the surface. In the Abitibi belt to the south, a comparatively non-reflective upper crust down to 3.0 s (but with the notable exception of the Bell River igneous complex near CDP 600) overlies a moderately reflective middle crust. The lower crust exhibits diffuse reflectivity that continues to 13.5 s in places before dying out. In marked contrast, the lower crust of the Opatica belt contains well defined, high-amplitude subhorizontal seismic layering and a sharp reflection Moho. The upper and middle Opatica crust contains a broad zone of high-amplitude reflectivity with reflections of opposing dips

between CDP 2,500 and 6,000 that extend down to 9 s. We infer the presence of a lower-crustal decollement (D) corresponding to shallowly north-dipping reflections between CDP 1 at 7 s and CDP 5,000 at 12 s, because more steeply dipping overlying reflections sole out at this level. The major changes in orientation of the seismic line are indicated. *b*, Interpretation of the seismic section in a displayed at true scale (1:1) along the seismic line. The major crustal units are indicated: OP, Opatica crust; OP LC, Opatica lower crust consisting of strong subhorizontal reflectors; AB, Abitibi (sub-greenstone) crust interpreted partly by correlation with earlier seismic data^{15,25}; NVZ GB, greenstone rocks that form part of the Abitibi northern volcanic zone; NEM, Nemiscau (metasedimentary) crust; O, subcrustal unit, tentatively identified as a relict Archaean oceanic slab. CP, LOP, LRP, SOA, NRSZ, FEGB, as in Fig. 2. D as in *a*. Unmigrated stack, F-K migration and line migration sections (*a*) were all employed in making the interpretation.

would have occurred at relatively shallow depths (that is, above the depth of melting of hydrated peridotite) and partial melting of the slab leaving a refractory eclogitic residue might be favoured over melting of the mantle wedge³⁶. It is also possible that the shear zone developed above a descending slab would provide a conduit for melts to migrate upwards, and that the observed reflections arise from eclogite that crystallized from melts within the shear zone. Alternatively, fluid migration is likely to occur preferentially along such a shear zone and induce metasomatism that could produce minerals such as amphibole; these could generate seismic reflections within a peridotitic mantle³⁷.

By combining the seismic data with geochronology and structural mapping, we are able to propose a plate-tectonic model for the collisional process. The pre-collision (pre-2,693 Myr) Opatica crust seems to have possessed a reflective lower unit and to have been separated from the Abitibi by a north-dipping subduction zone, possibly associated with its formation. At an early stage of collision, the present-day greenstone belt, which has the geochemical signature of primitive oceanic crust¹⁷, was emplaced. Further thrusting of the Abitibi margin beneath Opatica displaced the lower crust of the latter to the north, creating the shallowly north-dipping lower-crustal decollement observed in the seismic data. The high-grade rocks of the central Opatica belt were uplifted late in the collision whilst still hot, and cooled at around 2,680 Myr ago in a low-pressure environment^{22,23},

consistent with this scheme. Following closure of the subduction zone, a remnant of the descending plate could have been trapped at the base of the newly assembled continental crust. After 2,686 Myr, movement of up to 60 km took place along the right-lateral NRSZ, imaged in the seismic data as a near-vertical boundary to ~8 s near CDP 7,200 between reflections of contrasting dips.

Thus, the collision that amalgamated the low-grade Abitibi and high-grade Opatica belts produced an Archaean suture zone with characteristics similar to those of more recent times. The seismic reflection data show that shortening was accommodated differently above and below a shallowly north-dipping decollement, and that the rapid exhumation of mid-crustal rocks in the Opatica orogen was linked to subduction into the mantle. The present orientation of the mantle reflections can only be indicative of the dip of the thrust in the final stages of the collision, and may not be representative of the dip at earlier times when shallower subduction could have occurred. □

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Speciation driven by natural selection in *Drosophila*

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REINFORCEMENT is the process by which natural selection strengthens sexual isolation between incipient species, reducing the frequency of maladaptive hybridization and hence completing reproductive isolation. Although this model of speciation was once widely accepted^{1,2}, its plausibility^{3,4} and experimental support^{5–7} have been recently attacked. Here we provide an example of speciation by reinforcement, in the North American fruitfly *Drosophila pseudoobscura*. The results suggest that females of *D. pseudoobscura* evolved increased sexual isolation from their sibling species, *D. persimilis*, by natural selection against maladaptive hybridization.

Hybrid female offspring of *Drosophila pseudoobscura* and *D. persimilis* are fertile, but hybrid males are sterile, providing a selective disadvantage to mismatching. Small numbers of hybrids have been captured in the field^{8,9}, showing that interspecific mating occurs in nature. *D. pseudoobscura* ranges across the United States from Texas to the Pacific coast. The range of *D. persimilis* is completely contained within that of *D. pseudoobscura*, being found only in the Pacific coastal states¹⁰. *D. pseudoobscura* and *D. persimilis* flies were collected from two areas of California where they co-occur (sympatric populations), Mather and Mount St Helena; and *D. pseudoobscura* was also collected from Provo (Utah), Flagstaff (Arizona) and Tempe (Arizona), three areas where *D. persimilis* is not found (allopatric populations).

Females of *D. pseudoobscura* and *D. persimilis* discriminate against heterospecific males. Males, however, court the two species indiscriminately¹¹. Because reinforcement would only operate in areas where species hybridize, the theory predicts that, when tested with *D. persimilis* males, *D. pseudoobscura* females from sympatric populations should show stronger sexual isolation than *D. pseudoobscura* females from allopatric populations. There are no allopatric populations of *D. persimilis* with which similar comparisons can be made using *D. pseudoobscura* males.

The reinforcement hypothesis was tested by pairing individual *D. persimilis* males with individual *D. pseudoobscura* females derived from either sympatric populations or allopatric populations. In five of six comparisons, significantly fewer interspecific matings occurred with the *D. pseudoobscura* females derived

TABLE 1 Matings of *D. persimilis* males to *D. pseudoobscura* females from sympatric (S) and allopatric (A) populations

Male: Mather <i>D. persimilis</i>				
	Female	Total observed	Mated	Probability
(S)	Mather <i>D. pseudoobscura</i>	200	73	0.5
(A)	Tempe <i>D. pseudoobscura</i>	200	72	
(S)	Mather <i>D. pseudoobscura</i>	102	16	
(A)	Flagstaff <i>D. pseudoobscura</i>	102	63	<0.001
(S)	Mather <i>D. pseudoobscura</i>	100	18	<0.001
(A)	Provo <i>D. pseudoobscura</i>	100	49	
Male: Mount St Helena <i>D. persimilis</i>				
	Female	Total observed	Mated	Probability
(S)	Mount St Helena <i>D. pseudoobscura</i>	147	23	<0.001
(A)	Tempe <i>D. pseudoobscura</i>	147	51	
(S)	Mount St Helena <i>D. pseudoobscura</i>	107	28	
(A)	Flagstaff <i>D. pseudoobscura</i>	107	56	<0.001
(S)	Mount St Helena <i>D. pseudoobscura</i>	100	33	<0.001
(A)	Provo <i>D. pseudoobscura</i>	100	56	
Probability across comparisons = 0.031				

All stocks and experimental flies were kept at 21 °C with 12-h light/dark cycles. All crosses were done within 1½ years of the flies being collected. Population stocks were constructed by crossing 2–4 isofemale lines from each locality (as many as were available): Flagstaff *D. pseudoobscura* (3 lines), Mather *D. persimilis* (4), Mather *D. pseudoobscura* (3), Mount St Helena *D. persimilis* (4), Mount St Helena *D. pseudoobscura* (2), Provo *D. pseudoobscura* (4), Tempe *D. pseudoobscura* (4). Virgin flies used for the experiments were collected from the population stocks and aged for 8 days after eclosion to reach sexual maturity and receptivity. Single male and female pairs were then placed without anaesthesia in an 8-dram food-containing vial and confined for 24 h. To identify matings, the females' spermathecae and seminal receptacles were dissected and scored for sperm presence. All data were collected blind; the experimenter did not know if the flies derived from sympatric or allopatric crosses. The proportions of matings in sympatric and allopatric populations were compared with independent one-tailed Fisher's exact tests of independence, and the probabilities are given. To develop a statistical test of the entire table, one of the numbers from each comparison (either the number of matings with the sympatric females or allopatric females) was chosen with equal probability and summed over all six comparisons. This bootstrapping procedure was repeated 10,000 times to obtain a null distribution for the number of matings. The proportion of summations that was less than or equal to the total number of sympatric matings (191) is reported as a probability statistic.

TABLE 2 Comparison of *D. persimilis* male courtship of sympatric and allopatric *D. pseudoobscura* females

Female	Courtship element	Mean (s.e.)
Mount St Helena <i>D. pseudoobscura</i>	Courtship latency	81 (17.2)
	Number of courtships	7.0 (1)
	Number of attempted copulations	5.4 (0.7)
Flagstaff <i>D. pseudoobscura</i>	Courtship latency	61 (9.6)
	Number of courtships	5.9 (0.8)
	Number of attempted copulations	4.9 (0.7)

Flies for these experiments were collected and aged as described above. Single male and female pairs were placed without anaesthesia in 8-dram food-containing vials and observed for 10 min. Courtship latency was defined as the time in seconds from introduction of the male until the male performed the wing vibration characteristic of courtship²¹. Courtships were scored when the male performed a wing vibration towards the female or attempted to copulate. Each episode terminated when the male no longer oriented to the female for 5 s. Observations were concluded after 10 min, and each pairing was replicated 25 times.

TABLE 3 Matings of *D. pseudoobscura* males to *D. persimilis* females from populations where it is rare (R) or abundant (A)

Male: Flagstaff <i>D. pseudoobscura</i>			
Female	Total observed	Mated	Probability
(R) Mount San Jacinto <i>D. persimilis</i>	100	10	
(A) Mather <i>D. persimilis</i>	100	40	<0.001
(R) Mount San Jacinto <i>D. persimilis</i>	100	20	
(A) Mount St Helena <i>D. persimilis</i>	100	17	0.36

from the sympatric populations (see Table 1). The sixth comparison showed no difference. The overall results are consistent with the expectation of the reinforcement hypothesis.

To assess whether this pattern could have resulted from *D. persimilis* male preference for allopatric *D. pseudoobscura* females, rather than differences in female *D. pseudoobscura* discrimination, measures of courtship of *D. persimilis* males with *D. pseudoobscura* females from a sympatric population and an allopatric population were quantified (see Table 2). In both tests, males courted and attempted to copulate immediately, showing no significant difference in courtship of sympatric versus allopatric *D. pseudoobscura* females.

Another prediction of reinforcement is that it should be stronger in the rarer species, because rarity increases the probability of mating with the wrong species and hence increases the strength of selection to reduce hybridization¹². An isofemale line

of *D. persimilis* was collected in 1993 from Mount San Jacinto, where the species is very rare (C. Babcock and W. W. Anderson, personal communication). *D. persimilis* is about as common as *D. pseudoobscura* at Mount St Helena¹³, and is more common at Mather¹⁴. Thus the reinforcement hypothesis predicts that *D. persimilis* females from Mount San Jacinto should show more discrimination than *D. persimilis* females from Mather and Mount St Helena against mating with *D. pseudoobscura* males.

To test this prediction, individual *D. pseudoobscura* males were paired with individual *D. persimilis* females derived from Mount San Jacinto and with *D. persimilis* females from the populations where *D. persimilis* is common. Mount San Jacinto females mated with *D. pseudoobscura* males significantly less often than did Mather females (see Table 3), supporting the reinforcement prediction. The results were not significantly different for the Mount St Helena population, but this outcome, too, is expected because *D. persimilis* is not as common at Mount St Helena as it is at Mather.

This study addresses two common criticisms of all previous studies of reinforcement. First, previous studies have dealt almost exclusively with species that are not known to hybridize in nature or produce some fertile hybrid offspring^{5,6}. Unless these conditions are met, there is no chance for gene flow and the operation of reinforcement; indeed the absence of gene flow in sympatry means that the two taxa were already good species^{15,16}. Second, the pattern of greater mating discrimination in areas of species overlap, which is often used to support reinforcement, could also result from the fusion or extinction of less isolated populations in sympatry⁷. This explanation seems unlikely in these species because high levels of gene flow apparently exist between *D. pseudoobscura* populations¹⁷⁻¹⁹, reducing the possibility of populational fusion/extinction.

These results suggest that female *D. pseudoobscura*, and perhaps *D. persimilis*, evolved increased sexual isolation in response to the maladaptive results of mismatching with heterospecific males in sympatry. It is unlikely that genetic drift or some other selection pressure caused both sympatric populations to evolve higher mating discrimination without any similar changes arising in the three allopatric populations. It is possible that an unknown third variable could explain these results; for example, an ecological variable could simultaneously allow *D. persimilis* to coexist within the range of *D. pseudoobscura* and also select for aberrant mating behaviour within *D. pseudoobscura* that fortuitously reduces heterospecific mating. Such a possibility, however, seems rather *ad hoc*, as it could produce changes in either direction. Reinforcement is a more parsimonious hypothesis because the conditions for its operation are known to occur and the specific prediction of direction is satisfied in these results.

Studies of the genetics of the reinforcement effect can now be performed. Previous genetic studies of isolating mechanisms might describe genetic changes that occurred only after speciation was completed²⁰. Investigating the genetic basis of the increased sexual isolation in *D. pseudoobscura* provides the rare opportunity to study genetic changes directly involved in the completion of speciation. □

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Normal differentiation patterns in plants lacking microtubular preprophase bands

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It is generally accepted that polarized cell expansion and the strict control of division plane alignment are prerequisites for ordered spatial development in higher plants¹. This appears to be linked to the presence of cell walls, which immobilize the cells and fix their relative positions. In this context, the cortical cytoskeleton is thought to play a central role¹⁻⁶. Interphase microtubules are often aligned perpendicular to the growth axis and it has been proposed that they control cell expansion, probably in combination with the cell wall. Another cytoskeletal array, the preprophase band, has been associated with division plane alignment. This structure, which girdles the cell at the G2 phase of the cell cycle and at prophase, precisely predicts the future division site and probably fixes it. Here we describe different mutants in *Arabidopsis* that are unable to form these two cortical microtubular arrays. As expected, this defect is associated with irregular cell expansion and the inability to align division planes. Surprisingly, however, the mutations do not affect differentiation patterns: all cell types and organs are in their correct relative positions.

Six mutants with an apparently identical phenotype were isolated during a mutant screen for abnormal cell elongation in the progeny of ethyl methane sulphonate and T-DNA^{7,8} mutagenized plants (ecotypes Wassilewskaja and Columbia). Genetic analysis shows that the phenotypes are caused by recessive, mendelian nuclear mutations. There are two complementation

groups, *ton 1* (from *tonneau*, the French for barrel) represented by one allele, and *ton 2* represented by five alleles. Both mutations result in very similar phenotypes and we were unable to distinguish double mutants from single mutants. The mutations are recessive, as crosses with the wild type always gave wild-type progeny. Mutants grow as short, thick misshapen plants of 2–3 cm (Fig. 1). All organs are present in their correct relative positions (Fig. 1). The mutants are not fertile.

At the cellular level, the mutations affect cell elongation and division plane alignment. Cell shape is highly irregular in all tissues and most cells seem to be unable to orient their axes. This is associated with the inability to align division planes, and even cells in the embryo divide, following apparently random patterns (Fig. 1). In roots and hypocotyls, for instance, the ordered cell files and layers observed in wild-type plants are completely absent (Fig. 1). In spite of these aberrant arrangements of cells, all cell types that can be distinguished by visual criteria are at their correct relative positions. This is true for root hairs, root cap cells, meristematic cells, epidermal cells, pollen, and so on. In fact, this phenotype very much resembles the one described for *fass*^{9,10}, which is a shape mutant perturbed from the embryo stage onwards. We think it is therefore likely that one of the *TON* genes is identical to *FASS*.

To characterize further the role of these genes at the cellular level, we studied the microtubular organization in wild-type and mutant plants. In wild-type plants, the interphase cells show microtubular arrays that are perpendicular to the axis of cell expansion. Occasionally, in addition, more randomly oriented arrays were observed (Fig. 2). Many interphase cells and all prophase cells in both root and shoot apical meristems have preprophase bands (Fig. 2). This corresponds to observations made for many plant species^{1,5,6}. Differentiated cells can show microtubular arrangements that vary from flat helical to longitudinal or random. Root hairs always show longitudinal microtubules.

In mutant plants, microtubular arrangements are strongly modified. First, interphase microtubules in the root meristem

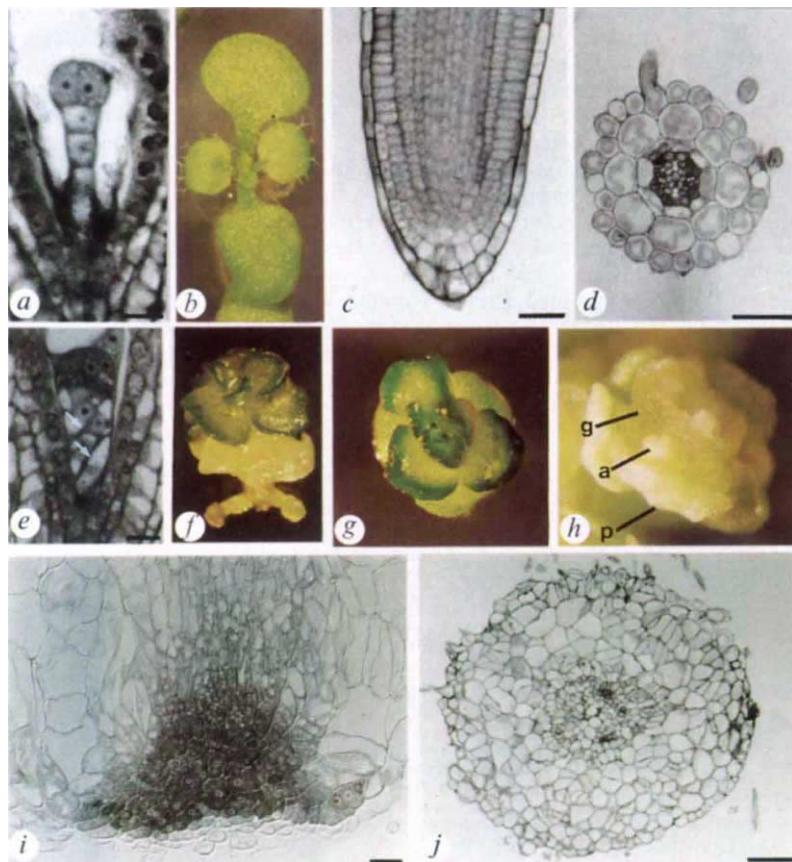


FIG. 1 Phenotype of wild-type (a–d) and *ton* (e–j) embryos and seedlings. a, Cross-section through an early embryo, showing suspensor cells arranged in a file and two cells of the embryo proper. b, Top view of the wild-type seedling. c, Cross-section through the root meristem showing cells arranged in ordered files. d, Cross-sections showing the simple anatomy of the root which consists of single-cell layers of epidermis, cortex, endodermis and pericycle surrounding the central cylinder (compare ref. 19). e, Section through the embryo of a mutant (*ton 1*). The cross walls are in random orientations. f and g, General phenotype of the mutant seedling *ton 2-1*. Plants are misshapen, but their leaves and roots are at their correct relative positions, which can be readily recognized. All flower organs are present (h); petals (p), anthers (a) and the gynoecium (g) can be seen. i, j, Cross-sections through the root apex show that cell shape in the meristem, and also in more mature tissues, is highly irregular. The typical cell files are absent. Cross-sections through the root (j) show that there are no distinct cell layers. Different tissues can be recognized, however, at their correct relative positions. Cells of the central cylinder can be recognized by their smaller diameter. The same is true for epidermal cells. Root hairs are also visible.

METHODS. For sectioning, seedlings were embedded in historesin (Leica, France) following the manufacturer's instructions. 5- μ m sections were cut on a Jung RM 2055 microtome and stained in 0.05% toluidine blue. They were examined in a Nikon microphot FXA microscope. Scale bars: a, 15 μ m; c, 25 μ m; d, 50 μ m; e, 15 μ m; i, 10 μ m; j, 60 μ m.

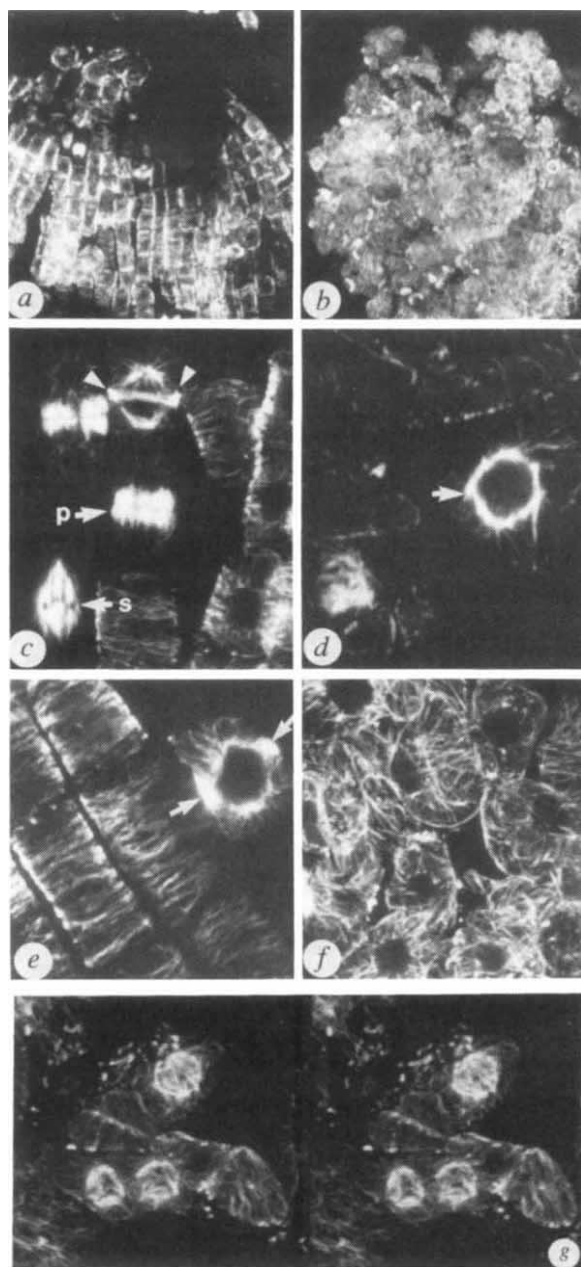


FIG. 2 Microtubules in root-tip cells of wild-type (a, c, e) and mutant plants (b, d, f, g; all from *ton 2-1*). Plants were fixed in 4% formaldehyde in 100 mM PIPES buffer, pH 6.9, containing EGTA (10 mM) and $MgSO_4$ (10 mM) and prepared as described²⁰. Root tips or apical meristems were partially squashed on poly-L-lysine-coated coverslips and air-dried. Cells were incubated with a monoclonal anti-tubulin (YOL 1/34; Sera Lab) and subsequently with FITC-labelled secondary antibody (goat anti-rat; Sigma). The cell cycle stage was determined using DAPI (Sigma) as a DNA stain at $1 \mu g ml^{-1}$. The preparations were examined in a Zeiss confocal scanning laser microscope using a 100 $\times$ objective. An argon laser provided the source of excitation light. Images were recorded using the software provided with the Zeiss system. Photographs were taken from a high-resolution flat screen monitor. a and b, Low-magnification optical sections through wild-type (a) and mutant (b) root tips, showing that cells often remained in their original relative positions. c, Wild-type meristematic cells with the typical microtubular arrangements of preprophase bands (arrowheads), spindles (s) and phragmoplasts (p); interphase cells usually show parallel microtubules (e). In the mutant preprophase bands were never observed. d, A prophase cell (DNA staining not shown) which still has cytoplasmic microtubules but, in contrast to the wild type, no preprophase band is observed. Interphase meristematic cells (f) always have random microtubules. g, Stereo pair of three prophase cells in the mutant with a forming spindle around the nucleus. All cells should have preprophase bands, but these structures are not detected.

seem to affect the cortical cytoskeleton, as spindle and phragmoplasts appear to form normally. In this respect the mitotic cells in *ton* mutants strongly resemble meiocytes and endosperm cells^{1,11}, which do not have preprophase bands and ordered cortical microtubules. Higher plants therefore appear to have the ability to switch the formation of these particular cytoskeletal arrangements 'on' and 'off'. The *TON* genes could be part of such a switch mechanism, and their isolation should provide us with important new tools to study the regulation of cytoskeletal organization.

In a more general context, our observations have important implications for existing models on morphogenesis in higher plants. These models all propose that both PPB formation (division plane alignment) and ordered cortical arrays of microtubules (directional expansion) are required for setting up the basic differentiation patterns of the development organs^{1,3,12-16}. Models on phyllotaxis, for instance, predict that changes in growth axis associated with leaf formation must be preceded by corresponding changes in the orientation of cortical microtubules and PPBs. The presence of PPBs has even been associated with the capacity of cell suspensions and non-organized callus to form organs^{17,18}. Our results, however, show that organogenesis and differentiation patterns can be set up without these specific cytoskeletal arrangements. They therefore support the idea that polarized cell expansion and division plane alignment are not required for the spatial positioning of organs and tissue types. □

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are no longer arranged in transverse arrays, but in random orientations instead (Fig. 2). Differentiated mutant cells never show completely ordered helical arrays, although locally microtubules can be parallel (not shown). A second major difference compared to the wild type is that preprophase bands are absent from the root and the shoot meristems, although spindles and phragmoplasts form normally. The same observation was made for preparations of 42 embryos and seedlings of the *ton 1* and *ton 2-1* mutants. We therefore conclude that the genes affected by the mutations are not only necessary for cell expansion and division plane alignment, but also for ordered arrangements of the cortical microtubules and for preprophase band (PPB) formation. Interestingly, the mutations do not affect tip-growing cells such as root hairs, which accordingly have a wild-type cytoskeletal arrangement (not shown).

Formally, it is unclear whether all characteristics of the phenotype described here are due to an aberrant organization of the cytoskeleton. Further characterization of the mutants and the *TON* genes is therefore necessary to assess the precise role of PPBs and interphase microtubules in the spatial development of higher plants. It is important to note that the mutations mainly

Hox gene expression in teleost fins and the origin of vertebrate digits

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Hox genes are essential for growth and patterning of the tetrapod limb skeleton¹⁻⁵. Mice mutant for the *Hoxd-13* gene have an important delay in morphogenesis owing to reduced proliferation². Based on the appearance of atavisms in such mice, we suggested that modifications of *Hox* gene regulation may have been a source of morphological variation during the evolution of tetrapod limbs^{2,6}. Pectoral and pelvic fins are homologous to fore- and hind-limbs, respectively. To compare the relative importance of *Hox* genes during fin versus limb morphogenesis, we cloned zebrafish (*Danio rerio*) *HoxD* and *HoxA* complex genes and analysed their expression during fin development. The results suggest a scheme for the fin-limb transition in which the distal autopods (digits) are neomorphic structures produced by unequal proliferation of the posterior part of an ancestral appendix.

Tetrapods organize their limbs by following similar developmental principles^{7,8}. This involves the production of a bud of mesenchyme from the lateral plate mesoderm, and its growth under the control of an ectodermal ridge, the AER. Consequently, structures appear progressively so that, for example, the humerus is produced before the wrist⁹⁻¹¹. While the skeletal pattern is laid down as a set of prechondrogenic condensations, posterior gene members of the *HoxD* complex (for example, *Hoxd-11* to *Hoxd-13*) are expressed in a biphasic manner. First, in budding limbs, transcripts are restricted posteriorly. Then, as the autopods (hands and feet) develop, *Hoxd* expression extends to most anterior and distal parts so that a switch from an anterior-posterior to a proximal-distal restriction is observed¹²⁻¹⁴.

Teleosts have highly apomorphic paired appendages^{15,16}, in which the endoskeletal part (homologous to the limb skeleton) has been modified in association with the development of a flexible dermal skeleton (Fig. 1), a structure that has been lost in tetrapods. This important difference in morphologies prompted us to characterize posterior *Hox* genes in the zebrafish (*Danio rerio*). DNA (40 kilobases) containing a region immediately 5' of *Hoxd-9* and extending upstream of *Hoxd-13* (that is, containing the *Hoxd-10*, *Hoxd-11*, *Hoxd-12* and *Hoxd-13* genes) was sequenced. The organization of the fish *HoxD* complex was similar to that of higher vertebrates.

Danio pectoral fin buds appear at 28–29 hours postfertilization as a thickening and outgrowth of patches of cells from the mesoderm overlying the yolk, facing the second and third somites (Fig. 1a). These mesodermal cells are surrounded by an ectodermal layer similar to that of tetrapod limbs, but the ectoderm soon protrudes and folds on itself¹⁷ (Fig. 1b). This fin fold progresses distally, and the dermal skeleton, the actinotrichia and lepidotrichia, appear inside the fold^{17,18}. Fold formation and the production of dermal skeleton coincide with an important reduction in cellular proliferation within the mesenchymal component. A few days after budding, two mesodermal sheets (~500 cells) are established. These cells barely divide until the end of the first week when they synchronously divide and start to condense (Fig. 1c). A cartilage model forms, except in the central part, so that two primary cartilages are observed, anteriorly and posteriorly (Fig. 1d). They split along the proximal-distal axis (Fig. 1e) to give the final four proximal radials¹⁹, identified as

R1 to R4, which form the basipterygium (Fig. 1f). Furthermore, smaller peripheral foci will generate the distal radials (Fig. 1e,f) which articulate with the lepidotrichia (Fig. 3). R1 to R3 contact the scapula (Fig. 1f), and R4 often articulates with R3 (Fig. 1f).

At 24 hours postfertilization, that is, before fin budding but after somitogenesis, *Hoxd-10* transcripts were detected as a uniform labelling in the mesoderm of the pectoral fin primordium, whereas *Hoxd-11*, *Hoxd-12* and *Hoxd-13* were not expressed. After 26 hours, *Hoxd-11* expression was restricted to the posterior half of the fin mesoderm, and some larvae expressed the *Hoxd-12* gene posteriorly. *Hoxd-13*, the most 5'-located gene of the complex, was activated after 28 hours, with expression being confined to posterior cells of the bud. As in tetrapods¹, collinearity was observed (Fig. 1g–k) and the ectoderm covering the fin buds (to become the fin fold) was negative.

Staining decreased in older stages. *Hoxd-10* was still expressed in fins after 40 to 46 hours, but expression was no longer observed subsequently, whereas *Hoxd-11* and *Hoxd-12* transcripts were found in the posterior half of the fin (Fig. 1l). In older fins (for example, after 52 hours; Fig. 1m), centrally located cells became negative so positive cells were found at the posterior margin and distally. However, in contrast to tetrapod limbs¹, very posterior-distal cells were negative. Consequently, after 58 hours, *Hoxd-12* expression was reduced to a subset of its early domain (Fig. 1n). This was also the case for *Hoxd-13* which, after 64 hours, was expressed in a few cells along the posterior margin as well as in a more posterior-distal position (Fig. 1o).

Posterior genes of the *HoxA* complex are also expressed in developing limbs. Unlike the *HoxD* genes, they show a distally restricted pattern without posterior preference^{4,13,14}. In particular, *Hoxa-11* is expressed at the autopod stage in an anterior to posterior stripe at the level of the distal zeugopod, with no expression in the distal autopod^{4,14} (Fig. 2d). In pectoral fin buds, *Hoxa-11* was expressed almost throughout the bud (Fig. 2a), in contrast to the posterior restriction of *Hoxd-11* (Fig. 1h). Later, the domain remained distal with positive cells both anteriorly and posteriorly (Fig. 2b). At the latest stage examined, expression appeared as a distal cap (Fig. 2c), in contrast to the situation in rodents^{4,14} or birds¹³ (Fig. 2d). However, the most distal cell layers were negative (Fig. 2c).

In actinopterygians, pelvic fins differ greatly from pectorals. Pectoral fins have an elaborate endoskeleton, crucial for the articulation and movement of the fin, whereas pelvic fins have short and simple elements and may thus be considered as abbreviated reiterations of the pectoral pattern¹⁵. Most elements do not articulate with the lepidotrichia, unlike homologous elements of pectoral fins (Fig. 3a,b). Expression of *Hoxd* genes was also observed in the posterior part of the budding pelvic fins (Fig. 3c), but in late pelvic fins the *Hoxd-12* domain did not reach the most distal cells. This domain was similar to that of the fish *sonic hedgehog* (*shh*) gene²⁰ (Fig. 3d), which organizes limb pattern in amniotes²¹. Therefore, even though the pelvic fins appeared ~30 days after the pectoral fins, *Hox* genes were reactivated together with the *shh* gene. In older fish the size of the pelvic fin mesodermal component did not increase significantly, so a correlation arose between the reduction of the pelvic fin endoskeleton, the rapid formation of a large fin fold (not shown) and a reduced expression of the *Hoxd* genes which were confined to a posterior patch of the developing fin mesoderm.

Comparison between *Hox* expression patterns in fins and limbs revealed striking similarities and differences (Fig. 4). In the budding phase, the expression of *Hoxd*, *Hoxa* and *shh*^{20,21} genes was similar in tetrapods and fish, which may reflect a conservation of the early developmental mechanisms involved. However, at later stages no expression of either *Hoxd-11*, *Hoxd-12* or *Hoxd-13* was detected in cells located in the anterior half of the fin, in complete opposition to the expression of the cognate genes in tetrapods², where anterior expression occurs in the late

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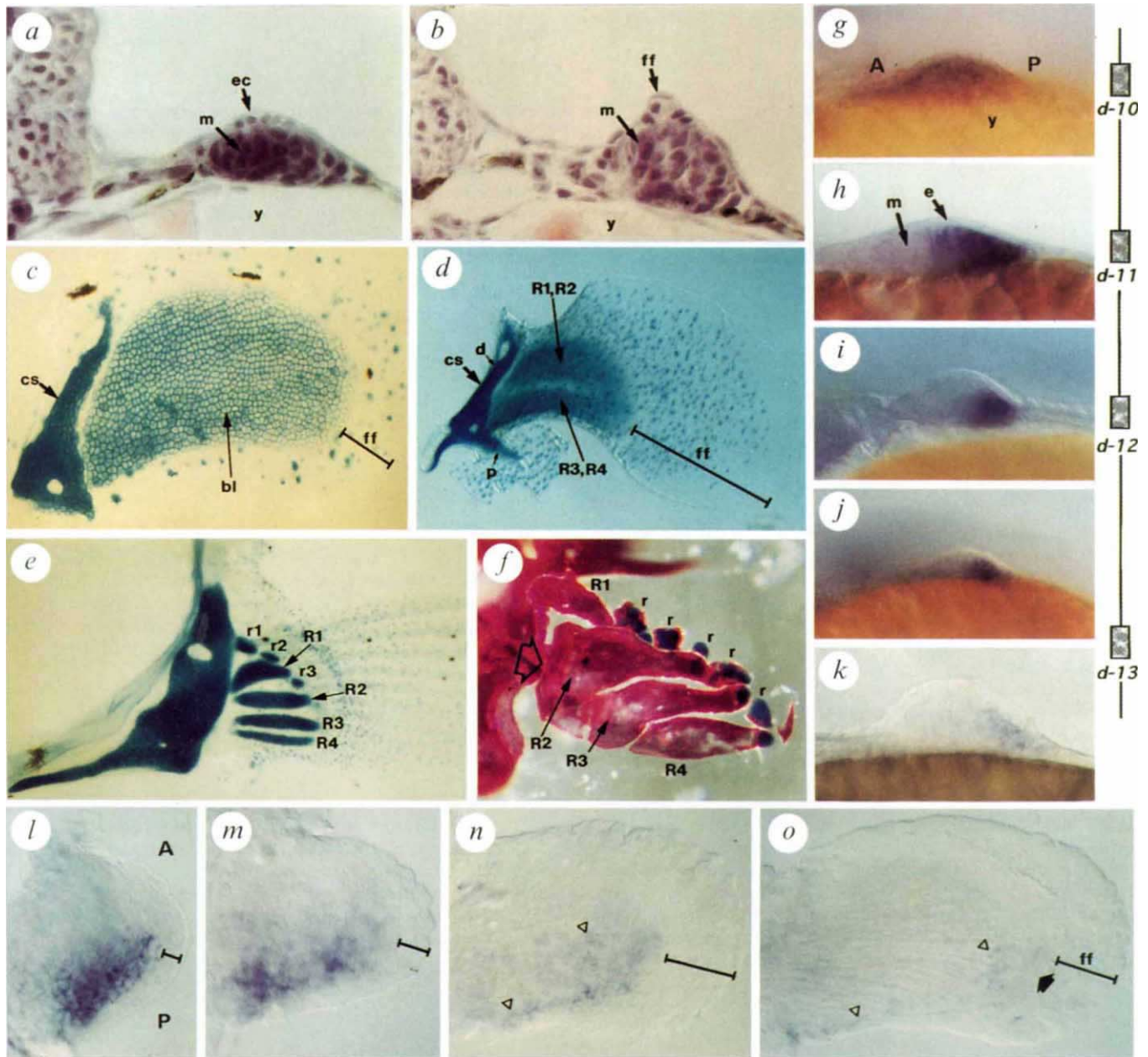


FIG. 1 Development of the zebrafish pectoral fin and expression of the posterior *Hoxd* genes. *a, b*, Sections through pectoral fin buds at the stages of *a*, the ectodermal ridge, ~28 h or *b*, early fin fold, ~30 h postfertilization. *c*, Dissected pectoral fin at an early stage of cartilage condensation (24 days postfertilization). The scapulocoracoid cartilage starts to condense. The fin fold (ff) is already well extended. *d*, After 28 days the two main condensations (R1, R2 and R3, R4) are formed as well as a large fin fold. *e*, By 38 days postfertilization the four proximal radials (R1 to R4) have separated. At the periphery, smaller condensations for the distal radials appear (r1 to r3). *f*, Dissected adult pectoral fin. The proximal radials (R1 to R3) contact the scapula (open arrow). Distal radials (r) are ossifying. The dermal skeleton was dissected out. *g–k*, Expression of the *Hoxd* genes at 34 h (*g–j*) or 40 h (*k*) postfertilization. Four consecutive genes are shown (right). *l–n*, Expression of the *Hoxd-12* gene at different stages of pectoral fin development. The black bar shows the extent of the fin fold. At 40 h (*l*), the expression is restricted to the posterior half of the fin. By 52 h postfertilization (*m*), expression is still uniform, although signal starts to weaken in central cells. Posterior-distal cells are negative. In older fins (58 h for *Hoxd-12* (*n*) or 64 h postfertilization for *Hoxd-13* (*o*)), expression has disappeared from proximal-central cells but remained in cells located at the posterior margin or more distally (arrowheads). Few cells are positive at

the posterior margin or distally (arrowheads), while most posterior-distal cells are negative (arrow). Abbreviations: ec, ectoderm; m, mesoderm; ff, fin fold; cs, coraco-scapular cartilage; d, distal process; p, proximal process; R1–R4, proximal radials; r, distal radials; A, anterior; P, posterior.

METHODS. For *in situ* hybridizations, animals were homozygous for the mutations *golden* (*gol*^{b1}) and *sparse* (*spa*^{b5}), and heterozygous for *albino* (*alb*^{b4}) (ref. 29). These triple mutants develop a weak pigmentation which facilitates detection of staining. Fish were randomly bred, and staging of the embryos was at 28.5 °C. For histology, embryos were stained with eosin-haematoxylin. For the observation of fins *in situ*, larvae were cleared, stained with alcian green (*c–e*) or alizarin red (*f*), dissected out and mounted on slides. A complementary DNA library was screened with murine *Hoxd* homeobox sequences. A fish *Hoxd-11* cDNA clone was used to screen a genomic library (λ fixII; Stratagene). Overlapping λ clones were isolated which encompassed a section of DNA starting about 20 kb 5' from the *Hoxd-13* gene and extending to the start of the *Hoxd-9* transcription unit. The entire locus was sequenced. Whole-mount *in situ* hybridizations used digoxigenin-labelled UTP, without acetylation and RNase steps³⁰. Embryos up to 34 h postfertilization were mounted in glycerol for observation, but older fins were removed and mounted separately.

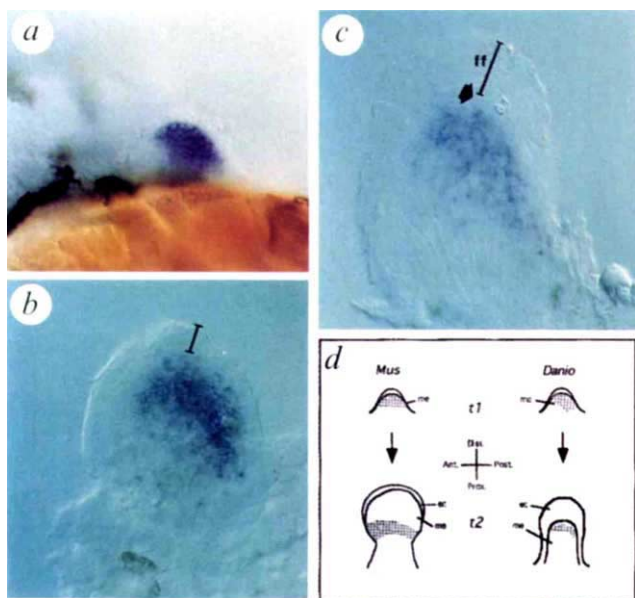


FIG. 2 Expression of the fish *Hoxa-11* gene during pectoral fin development. *a*, At the late budding stage (37 h postfertilization), *Hoxa-11* was expressed in both anterior and posterior distal cells. This was maintained at 48 h (*b*), and the distal domain still contained anterior and posterior cells at 60 h (*c*), by which time the most distal cells were negative (arrow). Soon afterwards, expression weakened and became undetectable after 100 h. *d*, Schematic comparison of the *Hoxa-11* expression between a mouse forelimb and a pectoral fin. A rather general expression in the murine bud was followed by a domain restricted to a band at the junction between the zeugopod and autopod. In teleosts, the late pattern was still confined to a very distal position with only a few distal cells negative. Data for the mouse are from refs 4 and 14.

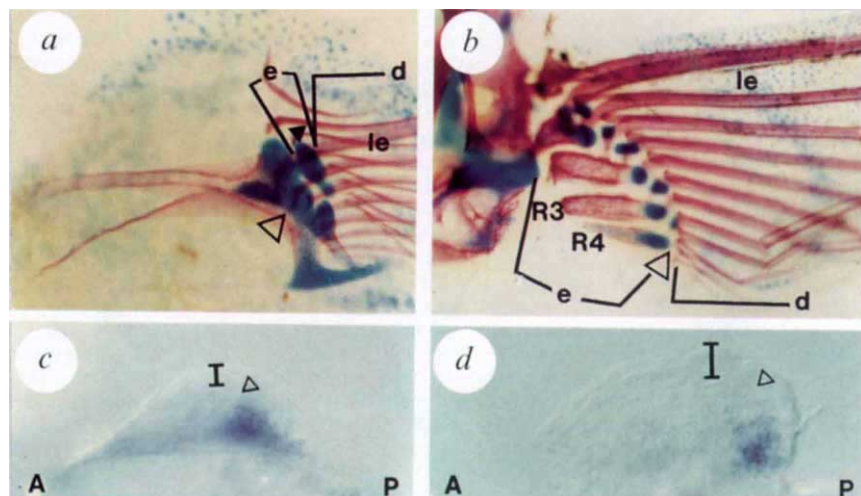
phase (Fig. 4*a–d*). Instead, the expression domains remained at the posterior margin of the fins, together with that of *shh* (not shown), and subsequently disappeared. Similarly, the late distal expression of the *shh* domain in tetrapods²¹, which may be due to signalling from AER²², was not seen in *Danio* and some positive cells remained close to the proximal posterior margin (not shown).

Proliferation of limb mesenchymal cells is controlled by the AER^{9–11}. The ridge releases factors which signal to the underlying cells to maintain a high mitotic state. In fins, the folding of the ectoderm and production of exoskeletal elements (actinotrichia) in between may mechanically interrupt the signalling and stop proliferation. The time at which such a transition occurs may therefore determine the relative extent of endo- versus ectodermal skeleton^{23,24} (Fig. 4*h*). For example, in the pelvic fins an early transition leads to both an extreme reduction in *Hox* and *shh* expression domains and an almost complete disappearance of endoskeletal elements. Tetrapod limbs do not show this epithelial transition and the structure proliferates until autopods are produced. Our results further suggest that these

terminal structures derive from the sustained proliferation of the posterior part of the limb^{14,24}. Such an unequal proliferation is illustrated by the dynamic of *Hox* gene expression domains in tetrapods (Fig. 4*a, c*), and may be controlled by asymmetrically distributed signalling molecules such as *Fgf-4* (ref. 25) in response to *shh*^{22,25} or the *Hox* genes themselves² through a feedback loop^{22,25}.

It has been suggested⁸, based on patterns of prechondrogenic condensations, that amniote limbs are built around a major axis which is bent anteriorly towards its distal part (at the level of the digital arch) and is homologous to the straight proximal–distal metapterygial axis of living or extinct fish species (see ref. 8; Fig. 4*e, f*). There is a correspondence²⁶ between the skewed *Hoxd* expression domains and the curvature of the axis²⁴. Our data on *Danio* reinforce this correspondence and suggest that vertebrate digits originate at the posterior margin. The straight fin axes of sarcopterygians such as *Eusthenopteron* (Fig. 4*g*) or the extant lungfish *Neoceratodus* indicate that such an unequal proliferation had not been achieved in these animals. Similarly, the striking difference in the fin versus limb *Hoxa-11* pattern

FIG. 3 Expression of the *Hoxd-12* and *shh* genes in the pelvic fin bud. The earliest sign of pelvic fin development was at ~30 days postfertilization, with the appearance of a cartilage model for the future basipterygium and a large fin fold. *a, b*, Morphologies of pelvic (*a*) and pectoral (*b*) fins. Pectoral fins have a rather elaborated endoskeleton (*e*), and the dermal skeletal elements (*d*) articulate with the distal and proximal radials (see open arrowheads on R4). In contrast, pelvic fins have much reduced endoskeletal elements (*e*), and the lepidotrichia articulate mainly with the basipterygium (open arrowhead) rather than with the radials (*e*). *c*, *Hoxd-12* expression in a budding pelvic fin (~30 days postfertilization). The expression is in posterior mesenchyme but is already restricted to proximal cells as most distal cells do not stain (arrowhead). *g*, Expression of *shh* in a late-budding pelvic fin. Expressing cells are confined to a posterior patch and do not extend to the fin fold (arrowhead). Abbreviations: *le*, lepidotrichia; *e*, endoskeleton; *d*, dermal skeleton; *A*, anterior; *P*, posterior; R3 and R4, third and fourth proximal radials.



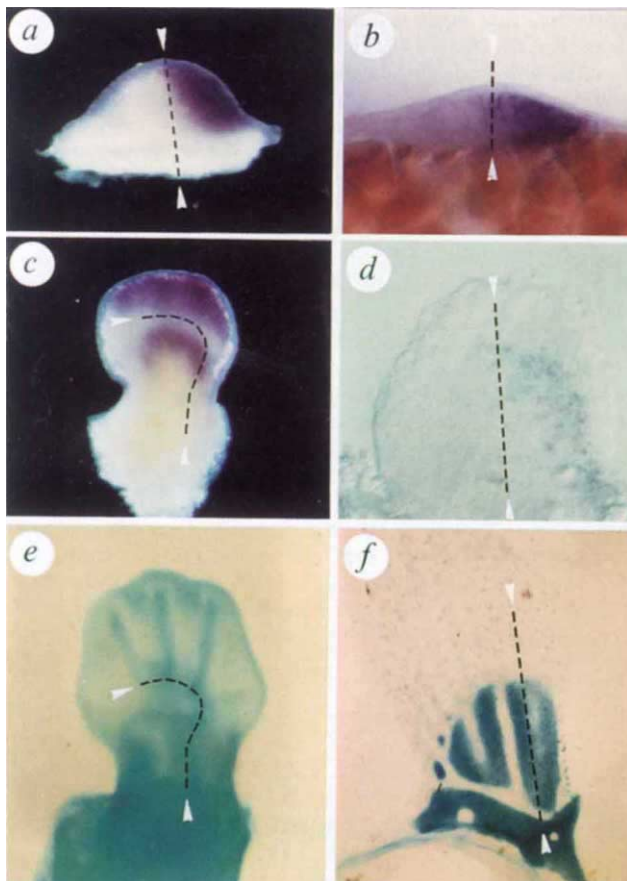
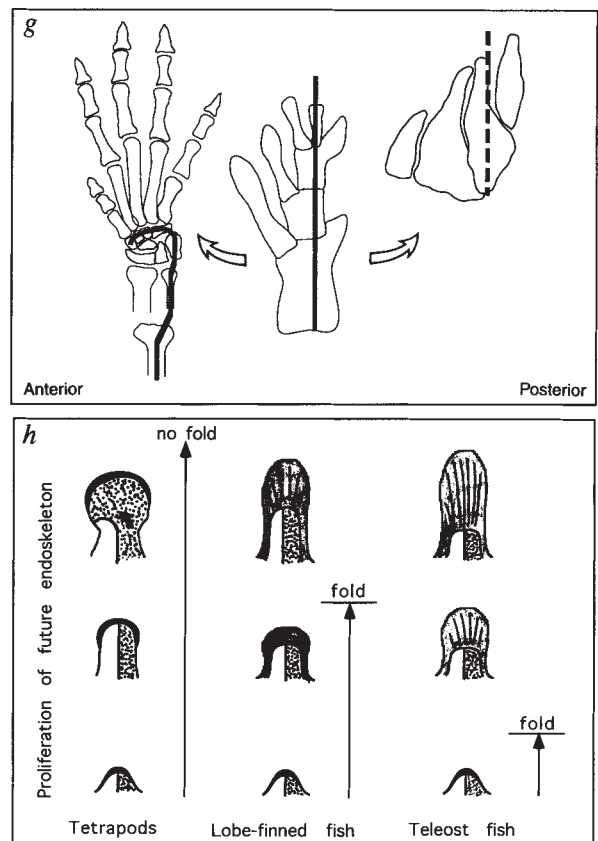


FIG. 4 Comparison of *Hoxd-11* expression in tetrapod versus fish paired appendages. *a, b*, In both mouse limb (*a*) and fish fin (*b*) buds, *Hoxd-11* expression was first restricted to the posterior half of the bud. Soon afterwards, however, these similar patterns diverge strikingly. *c*, In a day 12.5 mouse forelimb the domain was reinforced and extended into more anterior areas, leading to skewed expression boundaries. *d*, In contrast, an old pectoral fin showed a decreased expression of *Hoxd-11* and further restriction to the posterior margin without anterior extension. These differences coincide with the potential positions of the major appendicular axes at the stage of cartilage condensation (*e, f*, broken lines). These results support the view that the transition from fin to limb occurred concomitantly with a bending of the metapterygial axis. *g*, A mouse forelimb (left), the pectoral fin of the basal stem tetrapod¹⁵



Eusthenopteron (middle) and the endoskeleton of the *Danio* pectoral fin (right). The bold lines represent the positions of the major axes. In teleost fins the position of the axis is unclear but certainly extends straight from proximal to distal. *h*, The extent of endo- versus dermal skeleton may depend upon the time at which the transition from ridge to fold occurs. In such a scheme, folding of the ectoderm never occurs in tetrapods, but takes place early in teleost fish, with a concurrent large extension of the dermal skeleton. In lobe-finned fish, an intermediate situation may result from a late folding. The 'no fold' situation allows unequal posterior proliferation and subsequent digit formation to occur (arrow). The posterior half of the mesenchyme is stippled. METHODS. Mouse mid-gestation limbs (*e*) were stained with alcian blue.

illustrates the absence, in teleosts, of a structure homologous to the tetrapod distal autopod (Fig. 2*d*). In this context, autopods may be considered as neomorphic structures and digits as a vertebrate speciality (ref. 27; see also refs 16, 28). Accordingly,

the bending of the amniote metapterygial axis would reflect an unequal posterior proliferation rather than the transformation of an existing axial structure and re-organization of the various pre-existing elements. □

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Dendritic spines as basic functional units of neuronal integration

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MOST excitatory synaptic connections occur on dendritic spines¹. Calcium imaging experiments have suggested that spines constitute individual calcium compartments^{2,3}, but recent results have challenged this idea^{4,5}. Using two-photon microscopy⁶ to image fluorescence with high resolution in strongly scattering tissue, we measured calcium dynamics in spines from CA1 pyramidal neurons in slices of rat hippocampus. Subthreshold synaptic stimulation and spontaneous synaptic events produced calcium accumulations that were localized to isolated spines, showed stochastic failure, and were abolished by postsynaptic blockers. Single somatic spikes induced fast-peaking calcium accumulation in spines throughout the cell. Pairing of spikes with synaptic stimulation was frequently

cooperative, that is, it resulted in supralinear calcium accumulations. We conclude: (1) calcium channels exist in spine heads; (2) action potentials invade the spines; (3) spines are individual calcium compartments; and (4) spines can individually detect the temporal coincidence of pre- and postsynaptic activity, and thus serve as basic functional units of neuronal integration.

Two-photon fluorescence microscopy is particularly well suited for optical imaging of dendritic spines. In contrast to conventional microscopy, two long-wavelength photons are simultaneously absorbed and combine their energies to excite a fluorophore not normally absorbing at such a wavelength⁷. This permits the use of infrared excitation light, which penetrates deeper into highly scattering media like brain tissue⁸. In addition, because the two-photon absorption rate depends quadratically on the density of photons (light intensity), excitation is limited to the focal volume, resulting in reduced photodamage while providing optical sectioning equivalent to a confocal microscope but without any loss of fluorescence light due to a detector pinhole⁹. We imaged secondary and tertiary branches of dendrites in stratum radiatum from 90 CA1 pyramidal neurons filled with the calcium indicator calcium-green-1 by whole-cell perfusion^{10,11}. Dendritic spines were clearly resolved down to a depth of 150 μm below the slice surface and could be imaged at high resolution for up to 40 time-

FIG. 1 Subthreshold synaptic stimulation produces calcium accumulations restricted to individual spines. **a**, Fluorescence image (top) and line scan (bottom) of two spines. A line scan is a spatio-temporal image formed by successive scanning of the same line every 2 ms (arrowheads), displayed sequentially. Paired-pulse subthreshold synaptic stimulation (SY; 50 Hz) and a burst of 3 action potentials (AP; 50 Hz) produced transient fluorescence increases due to Ca^{2+} influx. Note that the right spine responds during APs, but not during synaptic stimulation. **b**, Images at different focal planes showing fluorescence intensity at rest (right panels) and difference between subthreshold synaptic stimulation (50 Hz, 100 ms) and rest (Δ , left panels), which highlights those pixels that increase in $[\text{Ca}^{2+}]$ during the stimulation. Bottom: projection of all images. Note that the only two spines that respond to synaptic stimulation (arrowheads) are in close proximity but located on different branches. **c**, Single spine activation under current clamp (synaptic stimulation: 40 Hz, 125 ms).

METHODS. We used a modified laser scanning microscope (Biorad MRC 600) with a Ti:sapphire laser with 100-fs pulses at 100 MHz (850 nm; Clark Instrumentation) and a 63 $\times$ 0.9 NA water-immersion objective (Carl Zeiss). Hippocampal slices from 14–30-day-old rats were cut with a vibratome and kept at 24–25 °C in a submerged incubation chamber³⁰. After 1–11 h, slices were transferred to a submerged recording chamber at 32 °C. Artificial cerebrospinal fluid (ACSF) contained (in mM): 124 NaCl, 5 KCl, 2 CaCl_2 , 2 MgSO_4 , 1.23 NaH_2PO_4 , 26 NaHCO_3 , 10 dextrose, with 95% O_2 /5% CO_2 . Sometimes APV (Sigma), CNQX (Tocris) or NiCl_2 (Sigma) were also included. Recordings³¹ were made with a patch-clamp amplifier (EPC-7; List Electronics) operating under voltage- or current-clamp (–70 mV). Electrodes were filled with 135 mM potassium methanesulphonate, 10 mM K-HEPES, 2 mM MgCl_2 , 0–5 mM $\text{Na}_2\text{-ATP}$ and 100–500 μM Ca-green; resistances were $\sim$ 7 M Ω . After break-in we waited 15–30 min before imaging. Electrophysiological signals were recorded using the second input channel of the scanning microscope.

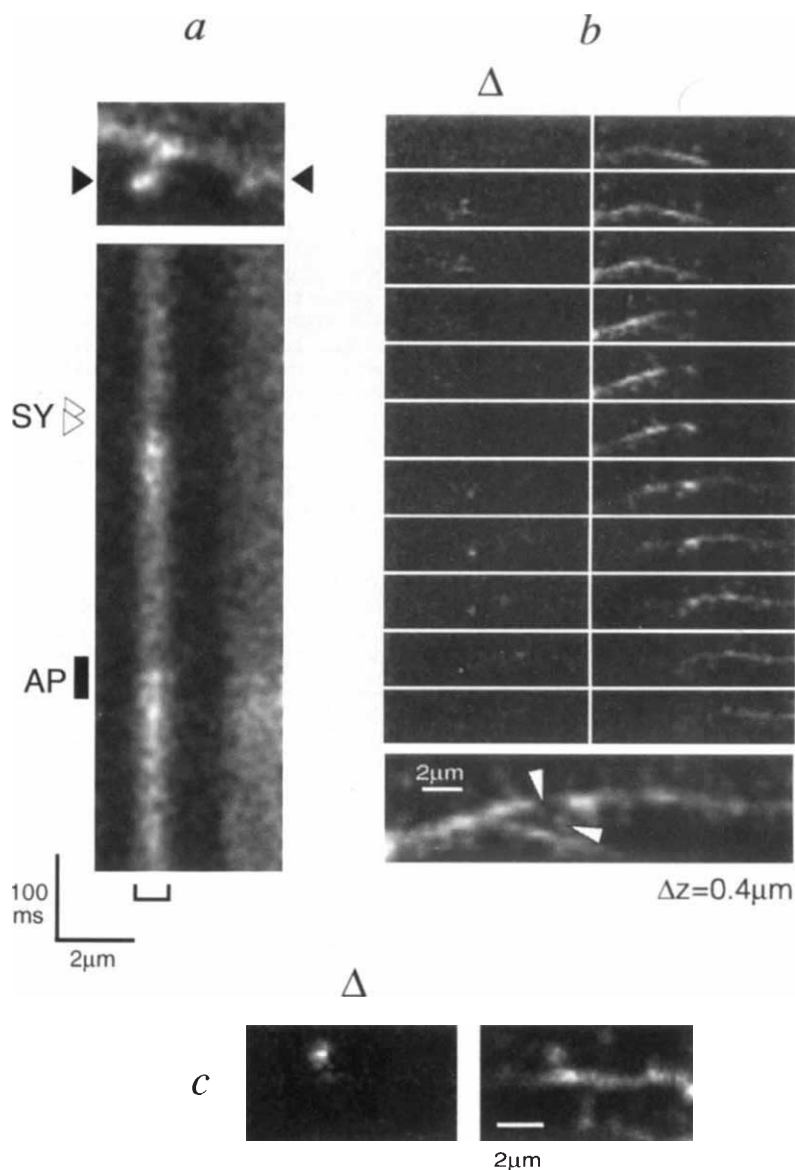
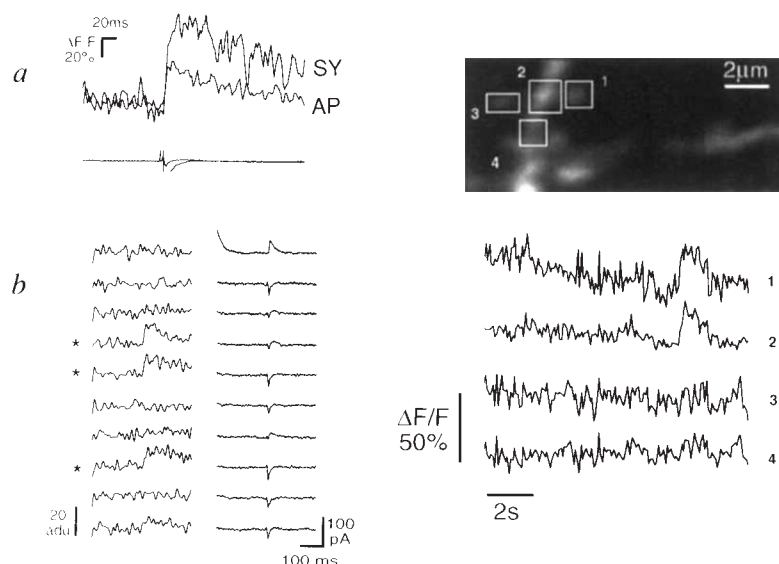


FIG. 2 Synaptic responses show stochastic failures and are similar to spontaneous synaptic events. *a*, Time course of the fluorescence response of a single spine (same as Fig. 1a) averaged from 5 trials (out of 19) that showed clear responses to single shocks compared with an average of 19 single-spike-induced responses from the same spine. *b*, Variability of synaptically induced Ca^{2+} accumulations in time-resolved line scans. Left, fluorescence measurements in the same spine in response to single-shock subthreshold stimulation (adu, digitizing units); right, simultaneous current recordings from the soma. Repeated stimulation with single presynaptic shocks produced variable calcium accumulations that can be classified as successes (asterisks) or failures. Data were smoothed to 8-ms time resolution. *c*, Spontaneous synaptic activity leads to calcium accumulations restricted to a single spine (1) and adjacent dendritic shaft (2), but not in the opposite spine (3) or more distal part of the dendritic shaft (4). Later during the same recording an accumulation restricted to the opposite spine (3) was seen (not shown). Recorded in movie mode (70 by 32 pixels at 13 Hz) in ACSF without Mg^{2+} (nominally), 4 mM Ca^{2+} , and TTX (5 μM).



resolved scans (2 ms per line; 512 lines) without signs of damage (Fig. 1a, top).

Focal synaptic stimulation, subthreshold for spike-initiation, induced transient increases of the free-calcium concentration ($[\text{Ca}^{2+}]_i$) restricted to spines and short portions of the dendritic shaft close to activated spines (Fig. 1). This was seen under both voltage- and current-clamp recording conditions (Fig. 1b, c). Spines adjacent to activated spines showed no detectable $[\text{Ca}^{2+}]_i$ increases even during trains of subthreshold synaptic stimulation (Fig. 1b, c). Synaptically induced $[\text{Ca}^{2+}]_i$ accumulations rose quickly (Fig. 2a), usually to more than half of their peak within 2 ms, the temporal resolution of the measurement.

As we could only find clear synaptically induced calcium responses to subthreshold stimulation when the stimulating electrode was less than 10 μm from the imaged dendrite, we investigated whether the changes measured were due to movement artefacts or to direct activation of voltage-sensitive channels by the stimulating current. We excluded both possibilities by using postsynaptic blockers. The combined perfusion of 2-amino-5-phosphonovaleric acid (APV) (100 μM) and CNQX (20 μM) completely abolished excitatory postsynaptic currents (e.p.s.cs) and calcium accumulation ($n=4$; not shown). As CA1 pyramidal neurons have $\sim 30,000$ spines¹² and threshold e.p.s.cs are

thought to require only 15–30 quanta¹³, our lack of success in finding active spines with remote stimulation can be explained by their scarcity.

Further evidence for a synaptic origin are stochastic failures of calcium to rise after single shock stimulation (Fig. 2b). As for e.p.s.cs¹⁴, the probability of failure (normally 0.6–0.8; Fig. 2b) dropped to almost zero with paired-pulse stimulation (results not shown).

Experiments on hippocampal cells in culture under conditions of spontaneous transmitter release have suggested that a unitary calcium compartment consists of a roughly 10- μm -long piece of dendrite (including all its spines)⁴. We tested whether the restricted calcium accumulation we found with synaptic stimulation also occurred when observing spontaneous activity (Fig. 2c) under high $[\text{Ca}^{2+}]_i$, zero $[\text{Mg}^{2+}]$ and tetrodotoxin (TTX), conditions that enhance spontaneous transmitter release¹⁵. Under these conditions, unlike in cultured neurons⁴, calcium accumulation is restricted to individual spines. This chemical compartmentalization is probably the result of the spine geometry, which impedes diffusional exchange between spine head and dendritic shaft¹⁶.

In contrast to the restricted pattern of $[\text{Ca}^{2+}]_i$ rise seen with subthreshold synaptic stimulation, a single postsynaptic action potential, triggered by a brief depolarizing pulse (3–10 ms, volt-

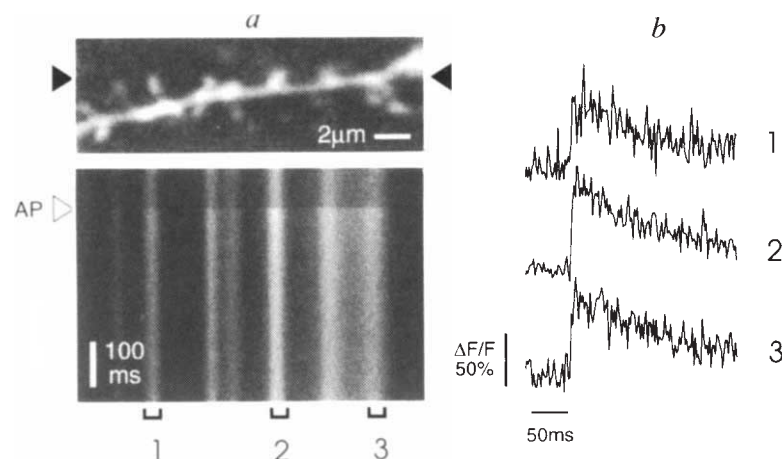


FIG. 3. Somatic action potentials induce calcium accumulations in all spines and dendritic shafts. *a*, Top, fluorescence image of a dendritic segment; bottom, line scan through 6 spines and nearby dendritic shaft (between black arrowheads) during the firing of an action potential in the soma triggered by a 3-ms, 50 mV voltage pulse (white arrowhead). All spines and the dendritic shaft respond simultaneously with significant calcium accumulation. *b*, The response to an action potential in spines and adjacent dendrites is similar. Time course of the fluorescence changes in two spines (1 and 2) and one nearby dendritic area (3) during the single action potential. Note the lack of significant delay between responses.

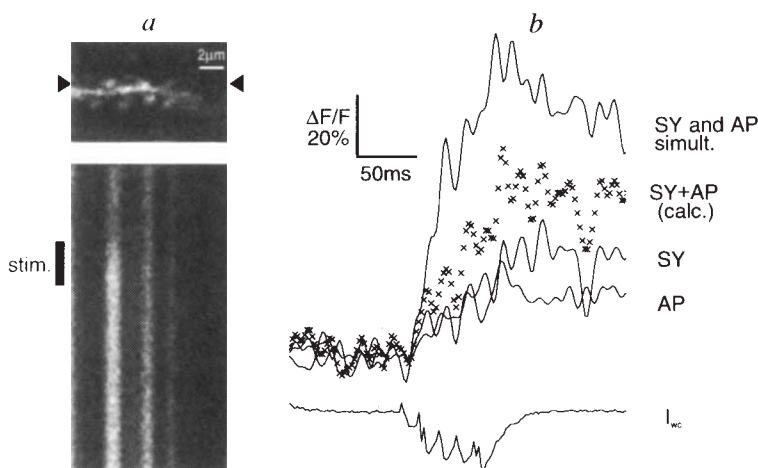


FIG. 4 The association of synaptic stimulation and post-synaptic action potentials produces supralinear calcium accumulations in spines. *a*, Top, fluorescence image of a dendrite; bottom, line scan through three spines (between black arrowheads) during a train of 5 subthreshold e.p.s.cs (75 Hz, stim.). Only the left spine shows calcium accumulations in response to synaptic stimulation (stim.). *b*, Top, time course of the fluorescence response of the left spine to e.p.s.cs (SY), postsynaptic spikes (AP) and their simultaneous combination (SY and AP). Averages of 2 stimulus presentations, which consisted of 5 e.p.s.cs or 5 spikes, produced by five 3-ms, 50 mV somatic voltage pulses, or the combination of both. Also shown for comparison is the calculated sum (SY+AP). In the neighbouring spine, responses to (SY+AP) and (AP) were identical (not shown). Responses were smoothed to 8-ms time resolution. Bottom, current trace during synaptic stimulation (I_{wc} , whole-cell current).

age or current clamp) at the soma, produced a rise of $[Ca^{2+}]_i$ in all imaged spines and dendritic shafts (Fig. 3). Action potentials triggered at the soma invade the dendritic tree of CA1 neurons¹⁷. Within the time resolution of our measurement (2 ms), we always found $[Ca^{2+}]_i$ to rise simultaneously in spines and adjacent dendritic shaft. These results extend previous measurements with slower time resolution¹⁸. The occasional spine that did not show spike-induced fluorescence increases always appeared comparatively bright, indicating an already high resting calcium level, presumably saturating the indicator.

The spike-induced calcium influx was due to opening of voltage-sensitive calcium channels (v.s.c.s) because it was reversibly abolished by perfusion of the v.s.c.c. blocker Ni^{2+} (2 mM; $n=2$), but it remained unchanged ($n=4$) with APV (100 μM) and CNQX (20 μM). Nevertheless, it is possible that calcium released from intracellular stores^{19,20} contributed to the later phase of the measured accumulations.

If v.s.c.s are located only on the dendritic shaft, we estimate, using published diffusion coefficients²¹, that it would take at least 30 ms for calcium to diffuse into the spine, even without any diffusional barrier at the spine neck. Therefore, particularly in light of the localization of synaptically induced calcium accumulation, the fast and ubiquitous calcium rise in response to action potentials shows that v.s.c.s are indeed located on spine heads, as well as on dendritic shafts, and that action potentials invade the spines.

Electrophysiological experiments imply that individual spines might detect the pairing of pre- and postsynaptic activity^{22–25}. Therefore we studied the interaction between spikes and synaptic input in single spines. We measured calcium dynamics in the same spine under three conditions: subthreshold synaptic stimulation, spikes, and both together. To avoid failures we used trains of synaptic stimulations. To reduce saturation and nonlinearity of the indicator, we loaded cells with a high (500 μM) concentration of calcium-green. Such a concentration reduces the absolute $[Ca^{2+}]_i$ levels attained, but still permits accurate comparison of influxes^{26,27}. We found that in most, but not all, spines the increase in fluorescence (F) resulting from simultaneous stimulation was supralinear, that is, it was larger than the combined responses to the individual stimuli (Fig. 4). Supralinear fluorescence increases were seen under both voltage-clamp (values of 177, 206, 145, 138, 152, 102, 103 and 101%) and current-clamp (134, 68, 106, 102, 110, 112%) recording conditions. As $F([Ca^{2+}]_i)$ is sublinear, or $F([Ca^{2+}]_1 + [Ca^{2+}]_2) < F([Ca^{2+}]_1) + F([Ca^{2+}]_2)$, a supralinear fluorescence response implies an even bigger supralinearity of the Ca^{2+} influxes.

Although the mechanism responsible for this $[Ca^{2+}]_i$ supralinearity is still not understood, it is clear that a single spine can use its $[Ca^{2+}]_i$ to register the temporal coincidence of the input and output of the neuron. Cooperative Ca^{2+} accumulation could then be used as a mechanism to target synaptic plasticity²⁸ or other calcium-dependent forms of chemical computation²⁹ specifically to individual synapses. □

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Inhibition of antigen processing by the internal repeat region of the Epstein-Barr virus nuclear antigen-1

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THE Epstein-Barr virus (EBV)-encoded nuclear antigen (EBNA1) is expressed in latently EBV-infected B lymphocytes that persist for life in healthy virus carriers^{1,2}, and is the only viral protein regularly detected in all malignancies associated with EBV^{3,4}. Major histocompatibility complex (MHC) class I-restricted, EBNA1-specific cytotoxic T lymphocyte (CTL) responses have not been demonstrated^{3,5}. Using recombinant vaccinia viruses encoding chimaeric proteins containing an immunodominant human leukocyte antigen A11-restricted CTL epitope, amino acids 416–424 of the EBNA4 protein⁶, inserted within the intact EBNA1, or within an EBNA1 deletion mutant devoid of the internal Gly-Ala repetitive sequence, we demonstrate that the Gly-Ala repeats generate a *cis*-acting inhibitory signal that interferes with antigen processing and MHC class I-restricted presentation. Insertion of the Gly-Ala repeats downstream of the 416–424 epitope inhibited CTL recognition of a chimaeric EBNA4 protein. The results highlight a previously unknown mechanism of viral escape from CTL surveillance, and support the view that the resistance of cells expressing EBNA1 to rejection mediated by CTL is a critical requirement for EBV persistence and pathogenesis.

EBNA1 is a phosphoprotein⁷ composed of unique amino- and carboxy-terminal domains (amino acids 1–89 and 327–641, respectively, in the prototype B95.8 EBV strain) joined by a repetitive sequence of Arg-Gly-containing motifs surrounding an internal Gly-Ala repeat⁸. Gly-Ala repeats of different length are present in all EBV isolates and represent the major target of EBNA-specific antibody responses⁹ but their function is unknown. We reasoned that presence of repetitive sequences covering over one-third of the molecule could influence the recognition of EBNA1 by the cellular immune system. Taking advantage of the observation that EBV induced CTL responses in caucasian human leukocyte antigen (HLA) A11-positive individuals are often dominated by A11-restricted reactivities to peptide epitopes corresponding to residues 399–408 and 416–424 of the EBNA4 protein^{6,10}, we constructed recombinant vaccinia viruses expressing chimaeric genes that contained the EBNA4 416–424 epitope inserted in-frame within the intact EBNA1 sequence, or within EBNA1 deletion mutants that did not contain the Gly-Ala repeats (Fig. 1a). Because of toxicity of the Gly-Ala repeats for vaccinia virus replication¹¹, only recombinants containing the Gly-Ala-positive chimaeras inserted in negative orientation relative to the vaccinia P7.5 promoter were selected. Expression of the inserts in negative orientation is likely to originate from a cryptic poxvirus promoter¹² resulting in delayed kinetics and relatively lower levels of expression. This allows vaccinia virus assembly, probably by counteracting the capacity of the repeats to compete for viral proteases that are required for maturation of virion proteins by cleavage at Ala-Gly-X motifs¹³.

Expression of the chimaeric proteins in human fibroblasts was confirmed by immunofluorescence (Fig. 1b, c) and immunoblotting (not shown). EBNA1 deletion mutants containing the EBNA4 416–424 epitope inserted at the His 39, Pro 446 or Lys 520 positions, relative to the B95.8 sequence (Vacc-E1ΔGAN-E4, Vacc-E1ΔGAP-E4 and Vacc-E1ΔGAB-E4, respectively) sensitized HLA A11-positive fibroblasts to lysis by EBV-specific CTLs (Fig. 2a). The level of killing was in each case comparable to that observed after infection with a vaccinia recombinant expressing EBNA4. In contrast, fibroblasts expressing a chimaeric full-size EBNA1 with the EBNA4 416–424 epitope inserted at His 39 (Vacc-E1N-E4) were not recognized by the CTLs (Fig. 2b). This was true even after infection for up to 24 h although the recombinant protein was detected in most infected cells starting from 12 h (Fig. 1c). Fibroblasts infected with Vacc-E1ΔGAN-E4 were lysed already after 6 h, and remained sensitive to lysis until 24 h when the experiment had to be terminated because of extensive cytopathic effects. The different recognition of cells infected with Vacc-E1ΔGAN-E4 and Vacc-E1N-E4 supports the view that presence of the Gly-Ala repeats may be directly involved in the failure of the E1N-E4 chimaera to sensitize target cells to lysis.

To explore this possibility further, we compared the CTL sensitivity of cells infected with vaccinia recombinants expressing EBNA4, or a chimaeric EBNA4 protein with the EBNA1 Gly-Ala repeats region inserted in-frame between Trp 624 and Pro 625, that is, downstream of the 399–408 and 416–424 epitopes (E4IR, Fig. 1a). Judging from the percentage of positive cells and intensity of fluorescence detected in experiments performed over a wide range of multiplicity of infection (m.o.i.), the recombinant proteins were expressed with identical kinetics and at similar levels (Fig. 1b, c). HLA A11-positive fibroblasts infected with Vacc-EBNA4 were sensitive to CTL clones specific for the 399–408 or 416–424 epitopes with maximal lysis after infection for 6 h (Fig. 3a) at a m.o.i. of 5 (Fig. 3c). In contrast, cells infected with Vacc-E4IR were lysed only weakly, even after infection at sixfold higher m.o.i. or for prolonged periods of time. Infection of the spontaneous lymphoblastoid cell line from donor QJZ (QJZsp LCL) that carries a Chinese EBV isolate with mutations abrogating recognition of the endogenous EBNA4 399–408 and 416–424 epitopes¹⁰ gave essentially similar results (Fig. 3b, d). Thus, the EBNA1 Gly-Ala repeats seem to affect processing and MHC class I-restricted presentation independently of the target cell lineage.

In the final set of experiments we asked whether overexpression of EBNA1 could by itself influence antigen processing. High levels of EBNA1 can be expressed in cells infected with vaccinia virus through the use of an inducible T7 RNA polymerase system¹⁴. HLA A11-positive fibroblasts infected for 12 h with Vacc-T7 and Vacc-EBNA1 or, as controls, Vacc-T7 and Vacc-EBNA3 were superinfected for 6 h with Vacc-EBNA4 (Fig. 4a) and then tested for sensitivity to lysis by CTLs specific for the 399–408 or 416–424 epitopes. Overexpression of EBNA1 or EBNA3 did not prevent recognition of the Vacc-EBNA4-infected fibroblasts although a 30 to 50% reduction of lysis was observed, probably due to competition of the viruses for the cellular transcription/translation machinery (Fig. 4b). Thus, presence of the repeats within the context of the target protein seems to generate a signal which either prevents processing or, alternatively, sequesters the processing products to a cellular compartment which is inaccessible to MHC class I-restricted presentation.

Additional studies are needed to clarify the mechanism by which the EBNA1 Gly-Ala repeats influence CTL recognition. Recent evidence suggests that processing of antigens for MHC class I-restricted presentation requires both ubiquitination¹⁵ and subsequent degradation by the proteasome¹⁶. The ubiquitin system has been implicated in the processing or complete destruction of many cellular proteins including proteins that are mutated, misfolded or otherwise damaged¹⁷. Our observation

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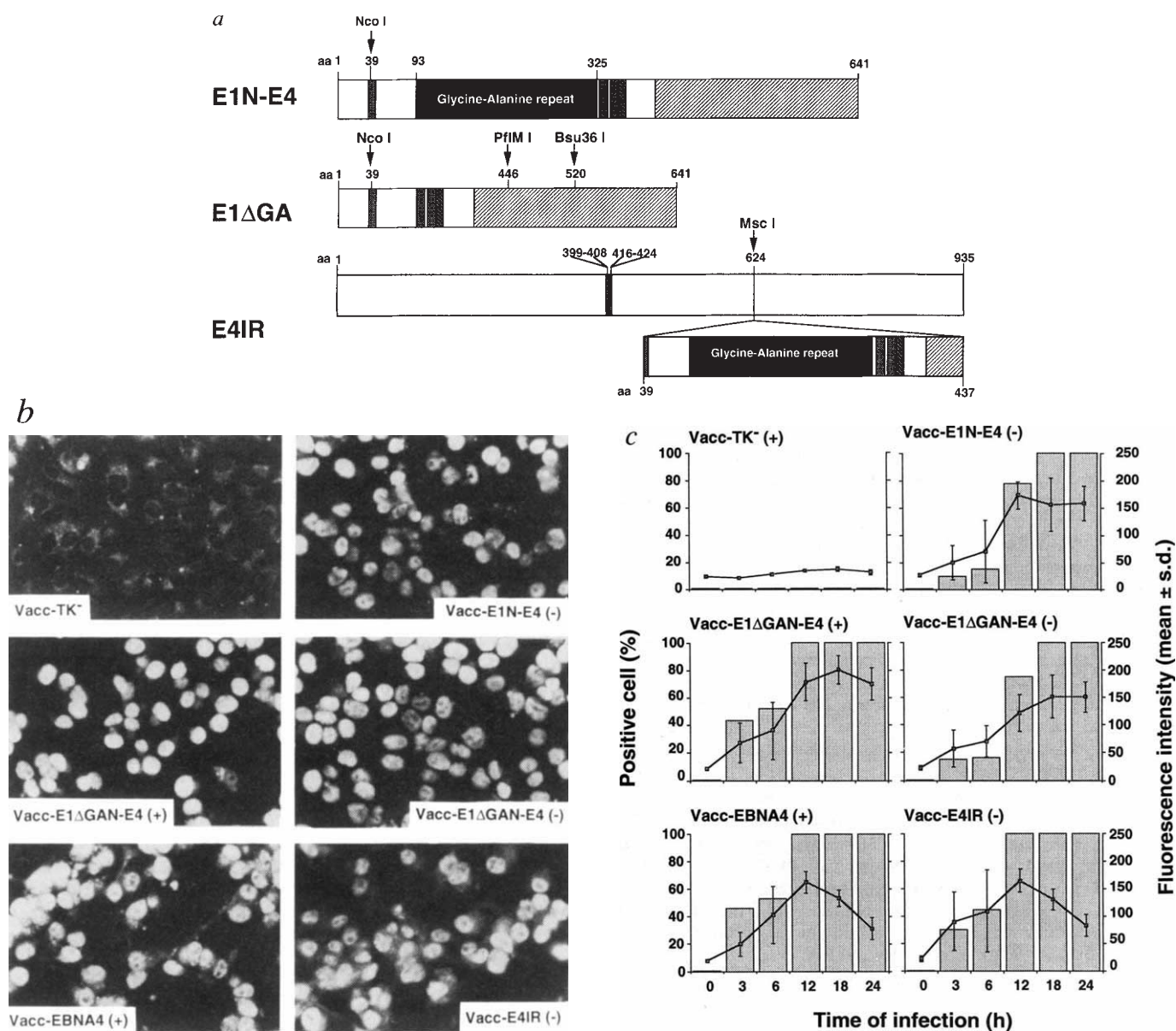


FIG. 1 *a*, Schematic outline of the full-length EBNA1 chimaera (E1N-E4), the Gly-Ala-deleted EBNA1 chimaera (E1ΔGA) and the Gly-Ala-containing EBNA4 chimaera (E4IR). The position of insertion of the EBNA4 416-424 epitope (E4) and the EBNA1 internal repeat region (IR) are indicated by arrows pointing to the amino-acid number in the B95.8 sequence. The EBNA1 Gly-Ala repeats (solid); Arg-Gly repeats (light shaded); nuclear localization signal (dark shaded); DNA binding and dimerization domains (cross-hatched) are indicated. *b*, ACIF stainings illustrating the nuclear localization of the recombinant E1N-E4, E1ΔGAN-E4 inserted in positive (+) or negative (-) orientation relative to the P7.5 promoter, EBNA4 and E4IR in vaccinia virus-infected fibroblasts. The E1ΔGAN-E4 and E1ΔGAB-E4 exhibited similar fluorescence patterns (not shown). *c*, Quantification of expression of the recombinant proteins detected by ACIF. E1N-E4 and E1ΔGAN-E4 reached a plateau of expression between 12 to 18 h whereas the expression of EBNA4 and E4IR reached a maximum at 12 h and decreased thereafter. The negative orientations E1N-E4 and E1ΔGAN-E4 were expressed with slower kinetics and at lower levels compared to the positive orientation E1ΔGAN-E4 whereas the orientation of insertion did not affect the expression of E4IR. It should be noted that the expression of E1N-E4 and E4IR is probably overestimated because of the presence of Gly-Ala-specific antibodies in the human serum.

METHODS. Vaccinia recombinants carrying the coding sequences of EBNA1 and EBNA4 (also known as EBNA-3B) from B95-8 were described previously¹⁴. Chimaeric proteins containing the EBNA4 416-424 epitope (IVTDFSVIK)⁶ within EBNA1 (E1), or an EBNA1 deletion mutant that lacks the Gly-Ala repeat region (E1ΔGA)¹¹ were produced

by inserting an oligonucleotide corresponding to the E4 epitope in the *Nco*I, *Pf*MI or *Bsu*36I site of the EBNA1 coding sequence (genomic positions: 108,067, 109,291 and 109,510, respectively). The oligonucleotide pairs E1N-E4F/E1N-E4R (5'-CATGCCATAGTAAGTACTGCTT-AGTGTAATCAAG-3'/5'-CATGCTTGATTACACTTAAGTCAGTTACTAT-3'); E1P-E4F/E1P-E4R (5'-CGATCGTAAGTACTGCTTGTAGTGAATCAGG-3'/5'-CCTTGATTACACTAAAGTCAGTTACGATCGTGC-3') and E1B-E4F/E1B-E4R (5'-TAACGATCGTAAGTACTGCTTGTAGTGAATCAAGG-3'/5'-TTACCTTGATTACACTAAGTCAGTTACGATCG-3') were annealed and ligated to appropriately digested pBS-E1 or pBS-E1ΔGA at 100:1 ratio. The chimaeric E1 and E1ΔGA open reading frames were excised by *Bst*YI and *Hin*CI digestion and inserted at the *Sma*I site of pSC11. The pBS-E4IR plasmic was constructed by inserting an *Nco*I-ApaI EBNA1 fragment (IR, gp: 108,067-109,261) into the *Msc*I site of EBNA4 (gp: 97,302). The E4IR open reading frame was excised by *Eco*RI and *Sst*I digestion and ligated into pSC11. Insert containing plasmids were sequenced to determine correct orientation and reading frame alignment. Recombinant viruses were generated by transfection into TK-143 cells infected with WR strain wild-type vaccinia and viral stocks were prepared and titrated in CV-1 cells¹⁴. Western blot analysis using a previously characterized human serum containing high antibody titres to all EBNA1 fragments (HR) confirmed that the E1N-E4 chimaera runs as a major band of 80K, corresponding to the full-length protein, and a 70K band, probably arising from recombination within the Gly-Ala sequence, whereas the three E1ΔGA chimaeras have a rough size of 52K (not shown). Attempts to detect the E4IR chimaera by immunoblotting gave inconsistent results, probably because of poor transfer of the 1,334 amino-acid-long product. The HR

that presentation of the E1N-E4 and E4IR chimaeras is inhibited rather than enhanced, as may be expected following modification of protein structure by insertion of foreign sequences, supports the notion that the Gly-Ala repeats may directly influence protein degradation.

MHC class I-restricted CTLs have potent antiviral activity both *in vitro* and *in vivo*^{18,20}. It is therefore not surprising that

viruses have evolved sophisticated mechanisms to escape CTL recognition. Previously identified routes of escape include suppression of target cell antigenicity by downregulation of MHC class I antigens²¹, or selective mutation of immunodominant CTL target structures^{10,22-25}; and modulation of the host immune response by clonal exhaustion or induction of immunosuppression^{26,27}. The results presented here highlight a

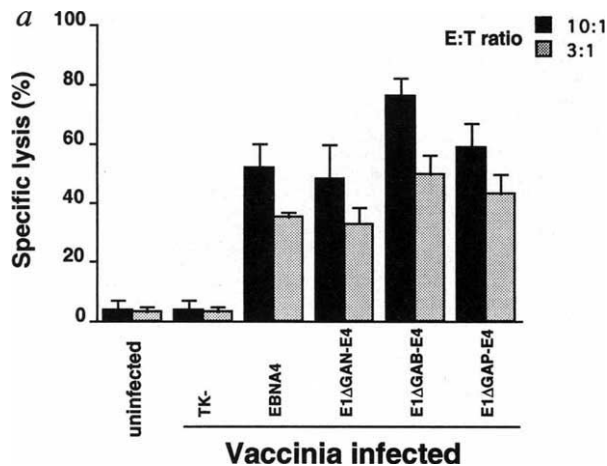
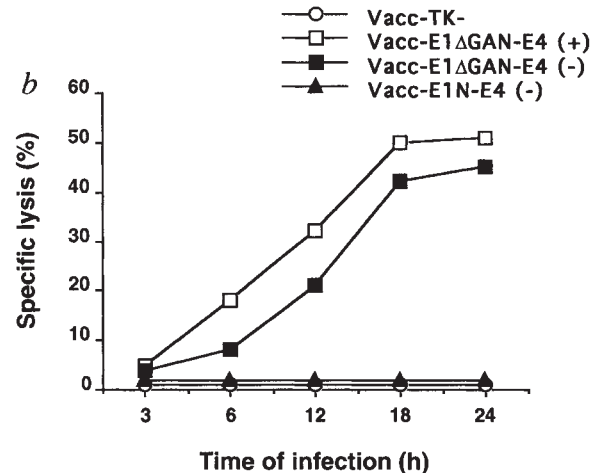


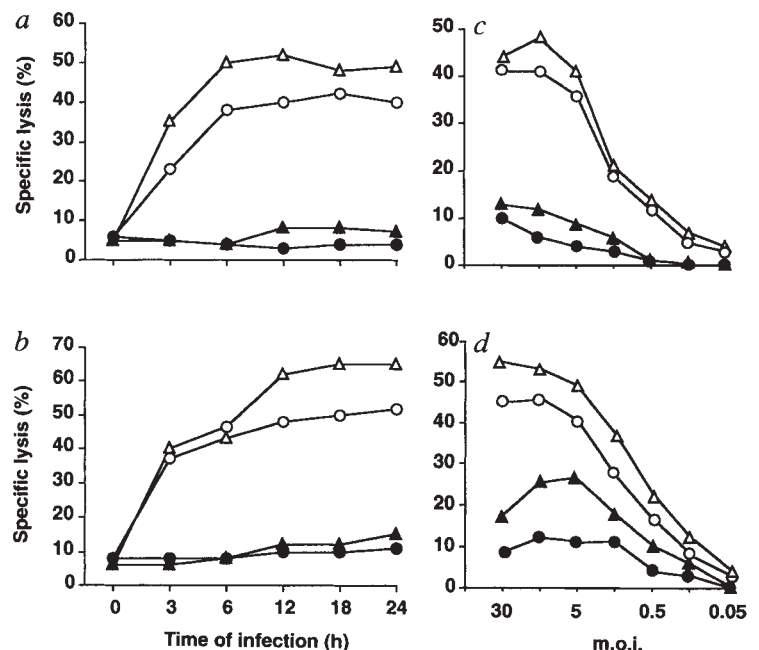
FIG. 2 Lysis of fibroblasts expressing the full-size and Gly-Ala-deleted EBNA1 chimaeras by CTLs specific for the EBNA4 416–424 epitope. Fibroblasts expressing the Gly-Ala-deleted chimaeras were lysed as efficiently as fibroblasts expressing EBNA4, whereas fibroblasts expressing the full-size EBNA1 chimaera were not killed. *a*, Lysis of fibroblasts expressing EBNA4, E1ΔGAN-E4, E1ΔGAB-E4 or E1ΔGAP-E4. Mean and s.e. of four experiments. *b*, Lysis of fibroblasts infected for different lengths of time with the Vacc-E1ΔGAN-E4 recombinant containing the Gly-Ala-deleted chimaera insert in positive (+) or negative (-) orientation Vacc-E1N-E4. Specific lysis at 10:1 effector:target ratio in one representative experiment out of three.



METHODS. Semiconfluent monolayers of fibroblasts from donor QJZ. (HLA A11 B13,B51) were grown in 96-well plates. Infection was done in the assay wells in the presence of 3 μ Ci 51 NaCrO₄ per well either for 12 h at a m.o.i. of 10 (*a*), or for the indicated times starting from 24 h before addition of the effectors (*b*). CTL clones specific for the EBNA4 416–424 epitope were obtained by stimulation of lymphocytes from the EBV seropositive donors BK (HLA A2,A11 B7,B35) with the autologous B95.8 virus transformed LCL as previously described⁶. The cytotoxic activity was assayed in triplicate in standard 4 h 51 Cr-release assays.

FIG. 3 Lysis of cells expressing EBNA4 or the E4IR chimaera by CTLs specific for the EBNA4 399–408 and 416–424 epitopes. QJZ fibroblasts (*a* and *c*) or the QJZsp LCL that carries a Chinese EBV isolate with mutations abrogating HLA A11-restricted recognition of the endogenous EBNA4 protein¹⁰ (*b* and *d*), were infected with Vacc-EBNA4 (Δ , $\circ$) or Vacc-E4IR ($\blacktriangle$, $\bullet$) at a m.o.i. of 10 for the indicated times (*a* and *b*) or at the indicated m.o.i. for 12 h (*c* and *d*) before use as targets for CTLs specific for the EBNA4 399–408 ($\circ$, $\bullet$) or 416–424 (Δ , $\blacktriangle$) epitopes. Specific lysis at 10:1 effector:target ratio in one representative experiment out of three done with each effector:target combination.

METHODS. Semiconfluent monolayers of QJZ fibroblasts were infected and labelled as described in Fig. 2. Aliquots of 5×10^6 QJZsp LCL cells were placed in 5-ml tubes and infected for 1 h at 37 °C with 1 ml concentrated virus before addition of 2 ml complete medium and further incubation for the indicated times. 100 μ Ci 51 NaCrO₄ were added to each tube 2 h before the initiation of the assay.



► serum was routinely used in complement enhanced immunofluorescence (ACIF) staining²⁸ to detect expression of the recombinant proteins in vaccinia virus-infected human fibroblasts. Parallel slides were stained with affinity-purified anti-Gly-Ala human antibodies⁹ and with the EBNA1-specific monoclonal antibody EBNA.OTx²⁹. For quantification experiments the fibroblasts were grown in 8-chamber slides. Separate chambers were infected at a m.o.i. of 10 starting from 24 h before the termination of the assay. Pairs of slides were stained with the HR serum

and with an EBV antibody-negative human serum as control. The percentage of positive cells was estimated visually using an epifluorescence microscope (LEICA, DMRB). Digital image recording was done with a cooled CCD camera (Hamamatsu C4880-01) controlled by the HiPic software. The images were analysed with the NIH Image1.56 software where fluorescence is proportional to the pixel value on a grey scale from 0 to 255. The mean fluorescence intensity of each sample was calculated from the pixel value of at least 50 nuclei.

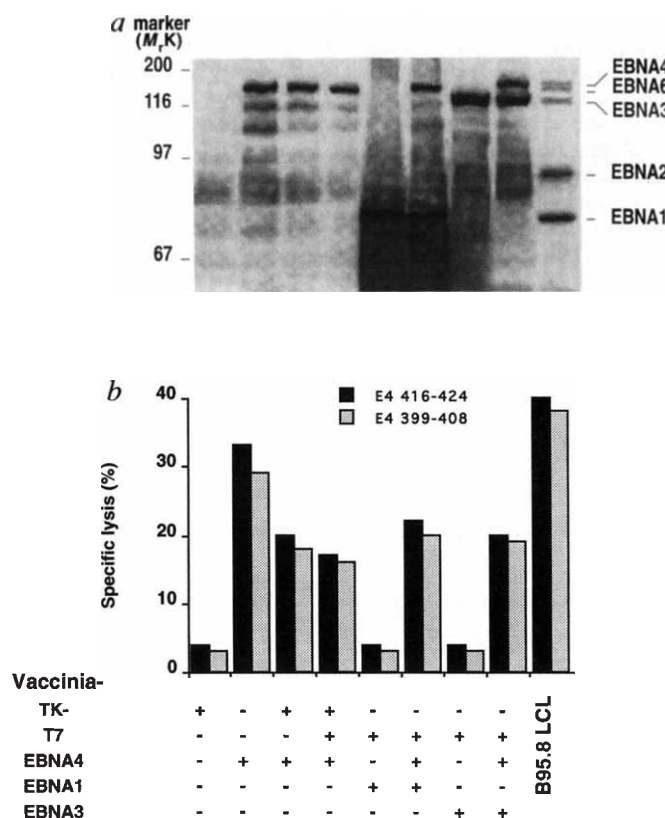


FIG. 4 Overexpression of EBNA1 does not inhibit presentation of the EBNA4 399-408 and 416-424 epitopes. **a**, Western blot illustrating the expression of EBNA4, EBNA1 and EBNA3 and coexpression of EBNA1 + EBNA4 and EBNA3 + EBNA4 in QJZ fibroblasts. The vaccinia-expressed EBNA3 and EBNA4 run as major bands of about 145K and 160K with minor degradation bands, whereas the T7 RNA polymerase-induced EBNA1 is seen as a major band of about 80K and a broad smear of immunoreactive material. **b**, Lysis of the same cells by CTLs specific for the EBNA4 399-408 (shaded) and 416-424 (solid) epitopes. Specific lysis at 10:1 effector:target ratio in one representative experiment out of three.

METHODS. The vaccinia recombinant carrying the coding sequence of EBNA3 (also known as EBNA-3A) from B95.8, and the T7 RNA polymerase-inducible EBNA1 expression system have been described previously¹⁴. QJZ fibroblasts were infected for 12 h with Vacc-EBNA1 + Vacc-T7, Vacc-EBNA3 + Vacc-T7 or Vacc-TK⁻ + Vacc-T7 before superinfection for 6 h with Vacc-EBNA4. Control samples were infected with Vacc-TK⁻ or Vacc-EBNA4 for 6 h. Each recombinant was used at a m.o.i. of 10. Western blots of 10⁶ infected cells were probed with the HR serum. A B95.8 virus-transformed LCL from donor QJZ was used as control.

new mechanism of immune evasion which is targeted to a particular stage of the virus-host interaction. Failure to undergo processing and MHC class I-restricted presentation of a constitutively expressed viral protein may afford protection independently of the class I type of the host. The high selectivity of this viral strategy may favour the long-term persistence of virus-infected cells without involving immunosuppression, thus maintaining the strong rejection-geared responses that prevent the uncontrolled proliferation of EBV-transformed immunoblasts. □

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Tilting of the light-chain region of myosin during step length changes and active force generation in skeletal muscle

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FORCE generation and relative sliding between the myosin and actin filaments in muscle are thought to be caused by tilting of the head region of the myosin crossbridges between the filaments¹⁻³. Structural and spectroscopic experiments have demonstrated segmental flexibility of myosin in muscle⁴⁻⁶, but have not shown a direct linkage between tilting of the myosin heads and either force generation or filament sliding. Here we use fluorescence polarization to detect changes in the orientation of the light-chain region of the head, the part most likely to tilt^{5,7,8}, and synchronized head movements by imposing rapid length steps⁹⁻¹¹. We found that the light-chain region of the myosin head tilts both during the imposed filament sliding and during the subsequent quick force recovery that is thought to signal the elementary force-generating event.

During muscle contraction, myosin crossbridges cycle through a series of biochemical, structural and mechanical states. We obtained partial synchronization of the transitions between these

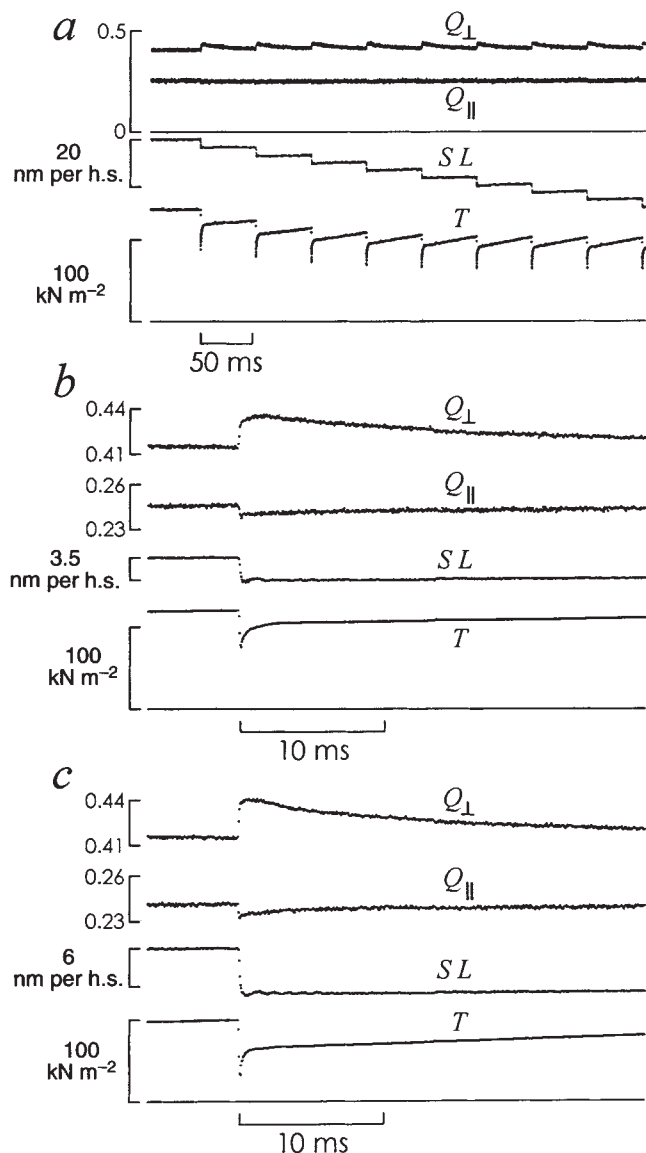


FIG. 1 Fluorescence polarization ratios (Q) from rhodamine probes on myosin regulatory light chains, sarcomere length change (SL , recorded in a separate activation), and tension (T), in a staircase of 150- μ s shortening steps imposed during active contraction²⁶ in (mM concentrations): TES, 100; CaEGTA, 25; $MgCl_2$, 6.9; Na_2 , ATP, 5.62; reduced glutathione, 10; creatine phosphate, 22.3; creatine phosphokinase, 250 U ml^{-1} ; free Mg^{2+} , 1; pH 7.06 at 12 °C. $Q_{\perp} = (I_{\perp} - I_{\parallel}) / (I_{\perp} + I_{\parallel})$ and $Q_{\parallel} = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + I_{\perp})$; the pre- and post-subscripts in the fluorescence intensities (I) indicate excitation and emission polarizations relative to the fibre axis. Fibre length, 2.26 mm; cross-sectional area, 5,500 μm^2 ; initial sarcomere length, 2.62 μm . a, Single activation; b and c, average response to 40 steps in 5 activations. Length steps: 3.5 nm per half-sarcomere (h.s.) in a, b and 7.1 nm in c.

METHODS. Wild-type chicken gizzard myosin regulatory light chain (RLC) was expressed in *E. coli*²⁷, labelled at Cys108 using 6-iodoacetamidotetramethylrhodamine²⁸, and purified on a Mono-S HR 5/5 FPLC column (Pharmacia) using a 0–400 mM KCl gradient. The peak eluting at 200 mM KCl was homogenous to >95% by reverse-phase HPLC. Endogenous RLC in single, glycerol-extracted fibres of rabbit *psaos* muscle was replaced by labelled RLC by 30-min incubation at 30 °C in EDTA rigor solution containing 0.7 mg ml^{-1} labelled RLC^{14,29}. Whole troponin and troponin C lost in this procedure were replaced. The motor³⁰, force transducer³¹, sarcomere length measurement³² and crosslinking of fibre ends^{33,34} have been described. Rhodamine fluorescence was excited by a 514 nm argon laser with polarization modulated at 84 kHz³⁵. Fluorescence intensities in line with the exciting light, polarized parallel and perpendicular to the fibre axis, were measured with photomultipliers and sampled at 500 kHz. $Q_{\parallel}$ and $Q_{\perp}$ were computed off-line with 40 μ s effective rise time (10–90%).

states by imposing a staircase of shortening steps at ~50-ms intervals during active contractions of single permeabilized fibres from rabbit *psaos* muscle (Fig. 1a). Each step caused a decrease of tension during the step, followed by a partial recovery within the next 1–2 ms^{9,12}. This quick recovery is generally considered to reflect the elementary force-generating event or working stroke in the myosin head^{3,11}. In the present experiments, rhodamine probes were introduced into the light-chain region of the myosin heads by replacing the endogenous regulatory light chain (RLC) with chicken gizzard RLC labelled at cysteine 108 (Fig. 1 legend). The measured fluorescence polarization signals ($Q_{\parallel}$ and $Q_{\perp}$) depend on the angle between the rhodamine absorption dipoles and the muscle fibre axis, and thus on the orientation of the light-chain region of the myosin head. Each length step shown in Fig. 1a produced an abrupt increase in $Q_{\perp}$ and a small decrease in $Q_{\parallel}$, showing that the rhodamine dipoles tilted away from the fibre axis. This was followed by nearly complete recovery before the next imposed step.

As the responses to each step in the staircase and in repeated activations were similar, they were averaged to improve the signal-to-noise ratio. The averages are shown on a faster timescale in Fig. 1b. $Q_{\perp}$ increased and $Q_{\parallel}$ decreased simultaneously with the length step; no time delay was detectable at the present 50- μ s time resolution. $Q_{\perp}$ increased further during quick tension recovery, and a two-exponential fit to the $Q_{\perp}$ signal after length steps averaging 3.0 ± 0.9 nm per half-sarcomere (mean $\pm$ s.d.);

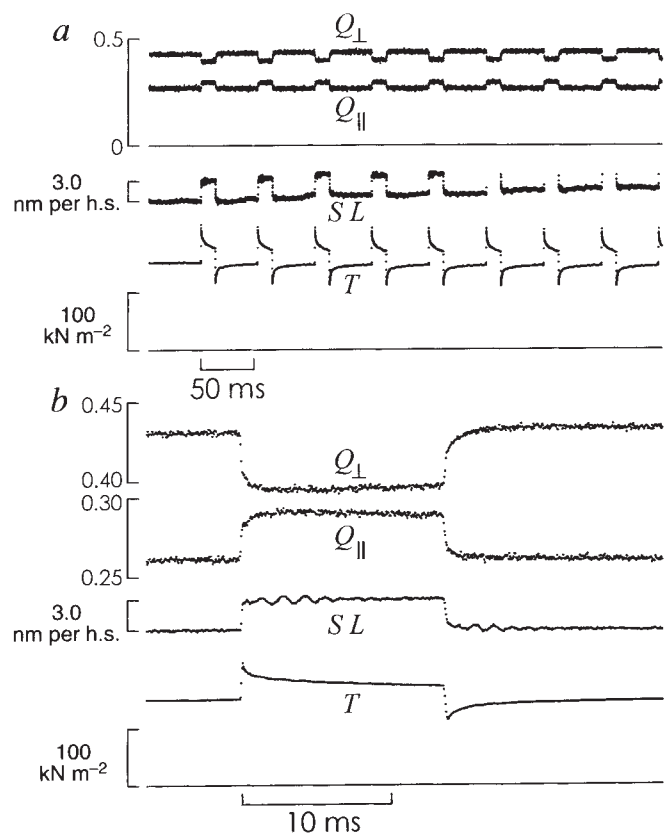


FIG. 2 Fluorescence polarization signals (Q), sarcomere length change (SL) and tension (T) in a series of quick stretches and releases during active contraction. a, Single activation; b, Q and tension signals from the average of 24 length steps recorded during 3 activations. Other conditions as in Fig. 1. In this experiment the sarcomere length signal saturated the digitizer after the first 5 cycles, and the average response from these 5 cycles is plotted in b. The transient oscillations in the SL signal are due to small sideways motions of the fibre. Fibre dimensions: length, 2.03 mm; initial sarcomere length, 2.46 μm ; cross-sectional area, 7,900 μm^2 .

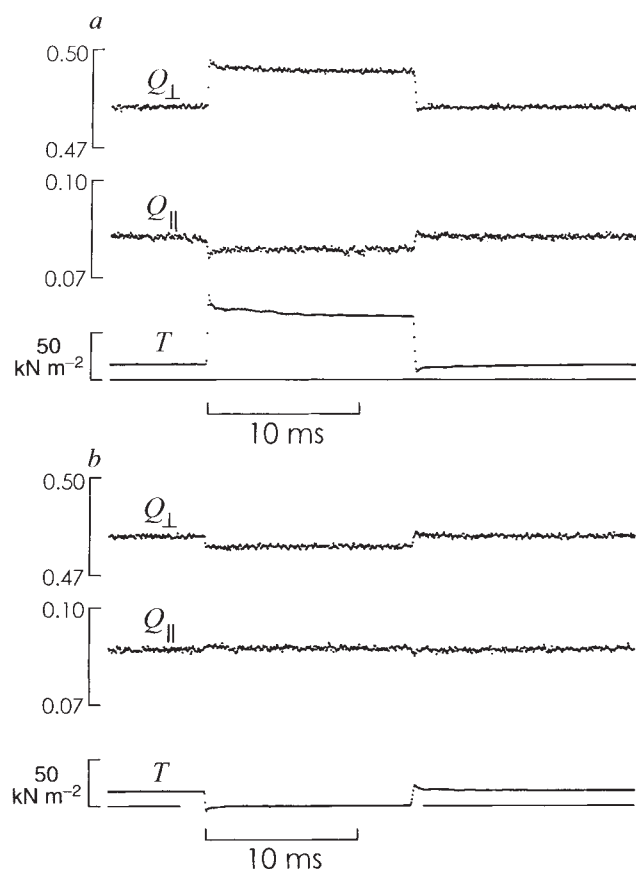


FIG. 3 Fluorescence polarization signals (Q) and tension (T) from a series of quick stretches and releases in rigor. *a*, Stretch first; *b*, release first. The sequence of solutions used to obtain rigor has been described²⁶. The rigor solution contained (concentrations in mM) TES buffer, 100; EGTA, 20; HDTA, 36.6; MgCl_2 , 1.8; reduced glutathione, 10; free Mg^{2+} , 1; ionic strength, 200; pH 7.06 at 12 °C. Traces are averages of 80 length steps of 3.4 nm per h.s. recorded with the stretch/release protocol described for Fig. 2, within a period of about 10 min during a single rigor contraction. Otherwise, conditions were as in Fig. 1. Fibre dimensions: length, 2.13 mm; initial sarcomere length, 2.58 μm ; cross-sectional area, 5,900 μm^2 .

$n=6$) gave rate constants of $1,690 \pm 210$ and $54 \pm 4 \text{ s}^{-1}$ for the rising and falling phases respectively. The rate constant for the rising phase is similar to that of the quick tension recovery, $1,530 \pm 110 \text{ s}^{-1}$ ($n=6$) for this size length step. When larger shortening steps of 7.1 nm per half-sarcomere were applied (Fig. 1c), the overall change in $Q_{\perp}$ was only slightly larger, showing that the peak change in the Q signals does not depend linearly on the size of the shortening step. Both tension recovery and the increase of $Q_{\perp}$ were faster after the larger step, and an extra

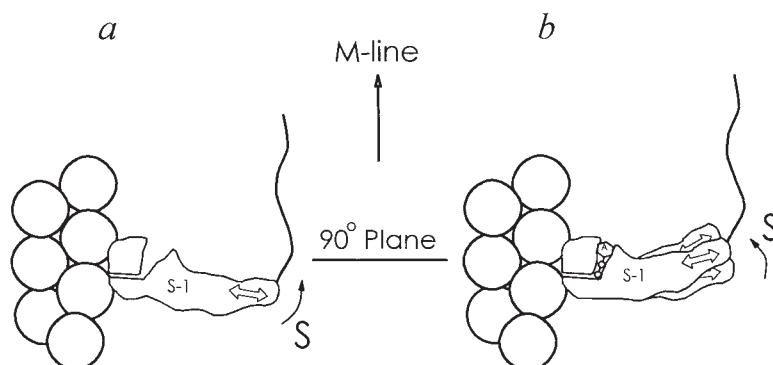
phase of recovery with a rate constant of about 400 s^{-1} was present in both polarization ratios (Fig. 1c).

The effect of a rapid stretch on the Q signals was studied by imposing alternate stretching and shortening steps in a cycle repeated at about 50-ms intervals¹³ (Fig. 2a). Each stretch produced a decrease in $Q_{\perp}$ and an increase in $Q_{\parallel}$, the opposite of the changes produced by shortening. The $Q_{\perp}$ and $Q_{\parallel}$ changes produced by a stretch had a similar amplitude, and both were reversed by applying a shortening step about 14 ms after the stretch. As the Q and tension responses were almost identical in each cycle, they were again averaged to improve the signal-to-noise ratio (Fig. 2b). $Q_{\perp}$ and $Q_{\parallel}$ changed simultaneously with the stretch, and further components accompanied the fast tension recovery. The Q and tension signals from the period between the stretch and shortening steps were fitted by an exponential plus a straight line. The exponential rate constant for $Q_{\perp}$ was $1,310 \pm 230 \text{ s}^{-1}$ ($n=5$), roughly similar to that for tension, $1,020 \pm 240 \text{ s}^{-1}$. The reversal of the Q signals by a shortening step 14 ms after the stretch also contained components during both the length step and the rapid tension recovery. For $Q_{\parallel}$, both components were much larger than those associated with a shortening step starting from the isometric state (Fig. 1).

These fluorescence polarization signals suggest that the light-chain region of the myosin head changes its orientation relative to the filament axis during the instantaneous elastic response to filament sliding, and that further tilting motions accompany the rapid transitions between states of the crossbridge cycle associated with active force generation. The latter transitions can be eliminated by removing ATP and putting the fibre in rigor. In rigor, $Q_{\perp}$ is much larger than $Q_{\parallel}$ (refs 14, 15), suggesting less orientational disorder of the rhodamine probes. When length steps were applied in rigor (Fig. 3), both Q signals and tension showed instantaneous elastic components, with little change after the length step, as expected for crossbridges attached to actin in the absence of ATP. These results support the interpretation of the Q changes during length steps in active contraction (Figs 1 and 2) on the basis of an instantaneous elasticity of the active crossbridge involving tilting of the light-chain region of the myosin head. Rhodamine probes on the most reactive sulphhydryl (Cys 707) in the catalytic domain of the myosin head do not change their orientation during similar length steps¹⁶. Thus the elasticity of the myosin head appears to reside either within the light-chain domain or between the light-chain and catalytic domains⁸.

Surprisingly, the polarization changes induced by length steps in rigor (Fig. 3) were in the opposite direction to those observed during contraction (Figs 1, 2). Stretching fibres in rigor tilts the probes away from the fibre axis; stretching contracting fibres tilts them towards it. This reversal of the instantaneous response suggests that the average probe orientations in the two states are on opposite sides of the plane at 90° to the filament axis (Fig. 4). The relative orientation of the dipole axis of the probes (double-headed arrow) to the long axis of the light-chain region of the myosin head is unknown. The simplest case is when the

FIG. 4 Cartoon representation of the myosin head (S-1), drawn after ref. 8, and the absorption dipole (double-headed arrow) of a fluorescent probe molecule attached to the regulatory light chain. In rigor (*a*) a stretch applied to the muscle pulls the light-chain region towards the M line, rotating the dipole axis towards the 90° plane. In active contraction (*b*), the probes are less well ordered, and a stretch S rotates the dipole away from the 90° plane, suggesting that the average dipole angle is on the opposite side of the 90° plane from that in rigor. Several conformations of the light-chain region are drawn in *b* to indicate its increased mobility during active contraction, which may be associated with the presence of different ATPase intermediates.



two axes are parallel, as shown in Fig. 4, and in this case our results would imply a large difference in the axial orientation of the light-chain region between the active and rigor states. If the probe axis is not parallel to the light-chain region, a rotation of this region about its long axis could explain the opposite response to stretch between the active and rigor states. This possibility seems unlikely, considering the structure of the myosin head bound to actin⁸, but cannot be excluded at present.

In conventional models for the mechanism of muscle contraction, force recovery after a shortening step (Fig. 1) is considered to be due to tilting of the myosin heads^{1,3}. Structural and *in vitro* motility data have indicated that the tilting might be in the light-chain region^{8,17,18}, and our results now provide direct evidence for this hypothesis. The angle change corresponding to the polarization transients in Fig. 1 can be estimated using a simple model of the orientation distribution of the rhodamine probes, consisting of an isotropic and a helically ordered fraction¹⁹. If all the probes in the ordered fraction (about 35% of the total) respond to the length step, the angle change is less than 3°, much less than expected in conventional contraction models^{1,3}. The discrepancy might be due to an unfavourable angle between the probe dipoles and the long axis of the light-chain region, but this seems unlikely because probes bound at other cysteine residues engineered into the RLC also give small *Q* signals (M.I., C.S.-D., Y.E.G., S. Hopkins, L. Saraswat and S. Lowey, unpublished data). Alternatively, only a small fraction of the myosin heads in the fibre might bear the isometric force and respond to the length step^{20,21}. This proposal seems difficult to reconcile with the high stiffness of active muscle^{22,23}, but would be consistent with the high force per myosin head measured *in vitro*^{24,25} and with several lines of evidence suggesting that the mechanical cycling rate in muscle fibres is faster than the average ATPase rate^{11,12,23}.

Regardless of the fraction of myosin heads involved in the fluorescence polarization changes we report here, the results provide strong evidence for tilting of the light-chain region during length changes and during force generation, two central tenets of the tilting crossbridge model. □

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Stimulation of E2F1/DP1 transcriptional activity by MDM2 oncoprotein

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THE MDM2 proto-oncogene is found amplified in a variety of tumours¹. The oncogenic capacity of the MDM2 protein is attributed to its ability to bind the p53 tumour-suppressor protein and mask its transcriptional activation potential^{2,3}. Here we show that MDM2 makes a functional contact with two cooperating transcription factors, E2F1 and DP1 (refs 4, 5), which are involved in S-phase progression⁶. MDM2 contacts the activation domain of E2F1 using residues conserved in the activation domain of p53. However, in contrast to its repression of p53 activity, MDM2 stimulates the activation capacity of E2F1/DP1. These results indicate that MDM2 not only releases a proliferative block by silencing the tumour suppressor p53, it also positively augments proliferation by stimulating the S-phase inducing transcription factors E2F1/DP1.

The MDM2 protein stimulates E2F1/DP1-dependent activation of an E2F site bearing promoter in U2OS cells (Fig. 1a). Under similar conditions, and in the same cell type, the MDM2 protein represses the transcriptional activity of the p53 protein (Fig. 1b), as previously shown^{3,7,8}. The stimulation of E2F1/DP1 by MDM2 is concentration dependent (Fig. 1c), and can be seen in other cell lines, such as SAOS2 and 208F (Fig. 1d and data not shown). Because SAOS2 cells lack functional retinoblastoma (Rb) protein and p53, our data indicate that the stimulatory effect of MDM2 is independent of Rb and p53.

The activity of the E2F1/DP1 heterodimer is due to an activation domain in E2F1. Figure 2a shows that there is a substantial degree of conservation (26% identity) between the E2F1 and p53 activation domains, which overlaps a region of p53 required to bind MDM2 (ref. 2). A short p53 peptide TFSGLW, shown to be essential for MDM2 binding⁹, is particularly well conserved in E2F1. The similarity in sequence also extends over a region of E2F1 and p53 required to bind TBP^{10,11}, which indicates that the observed similarities reflect a conservation of protein binding sites.

As predicted by the conservation in sequence, the activation domain of E2F1, like that of p53, binds to MDM2 *in vitro* (Fig. 2b). In addition, like p53 (Fig. 2c), the E2F1 protein binds MDM2 residues 1 to 220 (Fig. 2d). The interaction between E2F1 and MDM2 is direct, as it can be demonstrated with bacterially expressed proteins (Fig. 2e), and is specific, as MDM2 cannot bind other transcription factors, such as Elk1 or RAR, in this assay (data not shown).

The DP1 protein, which forms heterodimers with E2F1, can also bind the MDM2 protein *in vitro* (Fig. 3a). A central domain 59 to 331 appears to bind more avidly than the full-length protein, and residues 226 to 375 are sufficient to mediate the direct interaction between DP1 and MDM2 1 to 220 (Fig. 3b).

The DP1 protein can also be found complexed with MDM2 in the cell (Fig. 3c). To show an *in vivo* association we immunoprecipitated DP1 from NIH3T3 cells and, following western blotting, probed for the presence of the MDM2 protein using

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an MDM2-specific antibody. This procedure identifies a band of relative molecular mass 90,000 (M_r 90K) which comigrates with the MDM2 protein present in NIH3T3 cell extracts. The presence of a peptide used to raise the DP1 antibody inhibits the indirect immunoprecipitation of the 90K protein. Using a similar procedure we have failed to confirm the E2F1-MDM2 interaction, probably because of very low levels of E2F1 in the cell.

The E2F1 activation domain is contacted by the Rb protein, which represses the activation functions of E2F1 (refs 10, 12,

13). Figure 4a shows that the binding site for MDM2 lies directly adjacent to that of Rb. E2F1 residues 359 to 407 can independently bind MDM2 but not Rb, whereas residues 407 to 437 can independently bind Rb but not MDM2 (Fig. 4a; ref. 10). However, both MDM2 and RB can bind with higher affinity to the entire E2F1 activation domain (380 to 437), so the MDM2 and Rb binding sequences might overlap to some extent.

As predicted by the alignment in Fig. 2, mutagenesis of E2F1 residues (DF) corresponding to the p53 TFSGLF sequence severely affects binding to MDM2. In contrast, three other point

FIG. 1 MDM2 augments the activation capacity of E2F1/DP1. *a, b, d*, Transient transfections¹⁰ in the cell lines shown using 0.1 μ g CMV-E2F1 (ref. 15) and CMV-DP1 (ref. 5), CMV-MDM2 (ref. 1) or CMV-MDM2 220 to 491 (amount shown), 2 μ g of a target, 3 $\times$ E2F-CAT (ref. 15), 0.05 μ g CMV-p53 (ref. 16) with 2.5 μ g 13 $\times$ p53-CAT (ref. 17). The average of at least three independent experiments is shown. *c*, Western blot of U2OS cell extracts with anti-MDM2 antibody SMP14 (ref. 9).

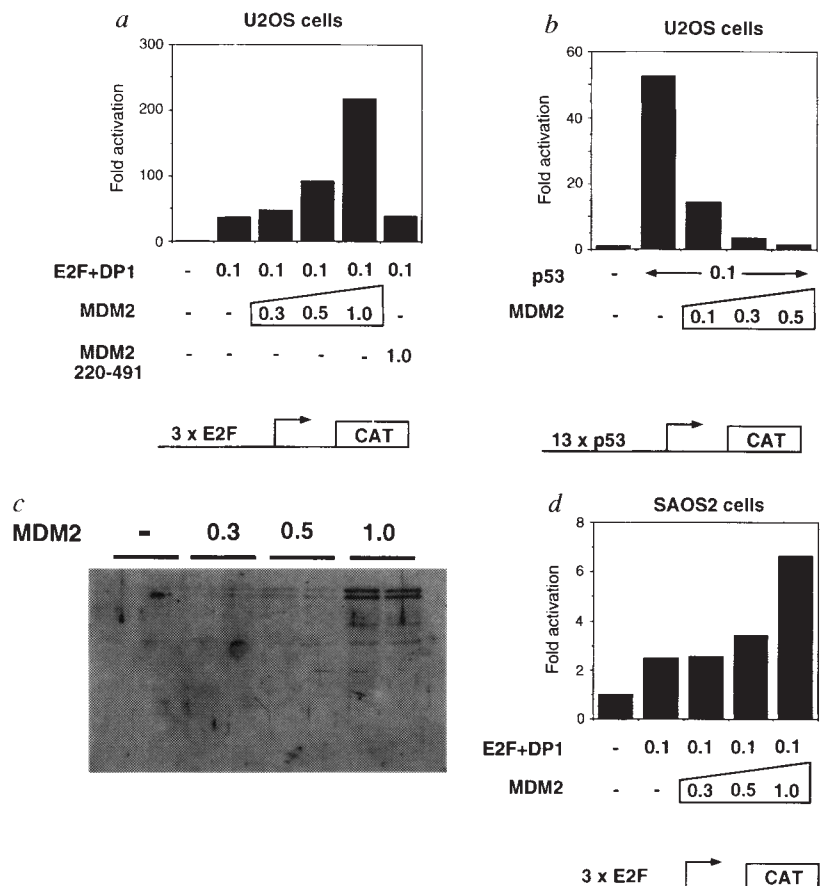
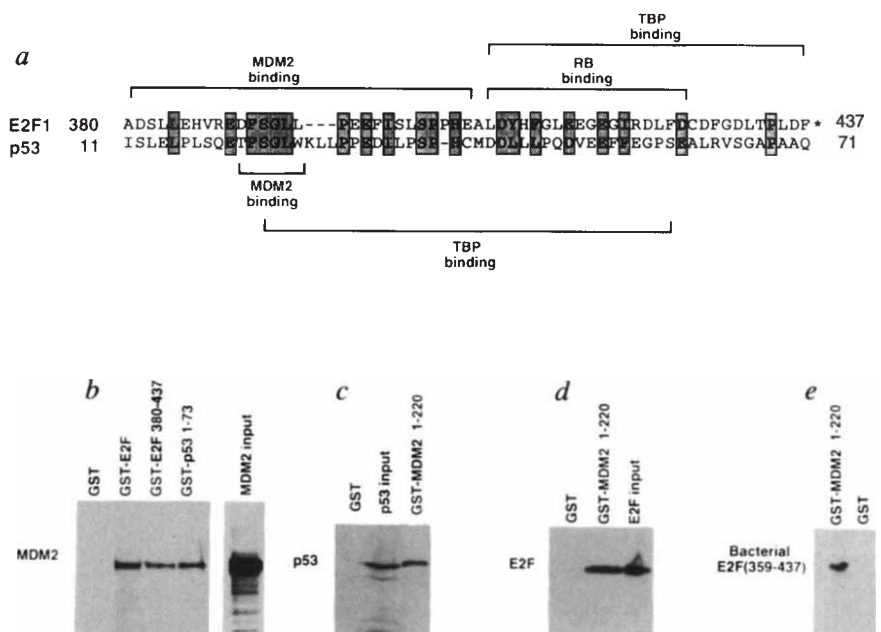


FIG. 2 Sequence similarity between the activation domain of E2F1 and p53 and their interaction with MDM2. *a*, Homologous residues are boxed. Horizontal bars indicate the regions of E2F1 required to bind Rb (ref. 18) TBP (ref. 10) and MDM2 (this paper) and the regions of p53 required to bind MDM2 (ref. 9) and TBP (ref. 19; mouse equivalent of human sequence). *b-d*, MDM2 (ref. 20), p53 and E2F1 proteins translated and radiolabelled *in vitro*, or *e*, radiolabelled after purification from bacteria, were subjected to a glutathione S-transferase (GST)-pull-down assay¹⁰.



mutations (HE, YF and DD) do not affect binding of MDM2, although YF prevents binding to Rb. These results confirm the validity of the alignment in Fig. 2 and suggest that the high-affinity binding sites for Rb and MDM2 are distinct.

If MDM2 can stimulate E2F1/DP1 activity as a result of a direct interaction, this *in vivo* stimulation is expected to depend on the respective domains of MDM2 and E2F1/DP1 required for *in vitro* interaction. Removing MDM2 residues 1 to 220 (required for the binding to E2F1 and DP1; Fig. 2 and 3) abolishes MDM2-mediated stimulation of E2F1/DP1 (Fig. 1a).

The reciprocal is also true (Fig. 4b, c) in that the MDM2 binding site in the E2F1 activation domain is required for this effect: MDM2 can stimulate the activity of the E2F1 activation domain (GAL4-E2F1 380 to 437) but will not stimulate a truncation (GAL4-E2F1 407 to 437) which lacks the E2F1 sequences required for MDM2 binding. These data support the conclusion that an MDM2-E2F1 interaction results in an increase in E2F1 activation capacity.

The mechanism by which MDM2 stimulates E2F1/DP1 activity is unclear. Considering that MDM2 has a domain that activ-

FIG. 3 DP1 interacts with MDM2. *a*, The MDM2 protein translated and radiolabelled *in vitro* (10% of input shown), or *b*, bacterially expressed and radiolabelled DP1, were subjected to a GST-pull-down assay¹⁰. *c*, An anti-DP1 antibody²¹ was used to immunoprecipitate DP1 from 600 µg NIH3T3 cell extracts²¹ in the presence of a peptide (50 mM) used to raise the antibody (lane 2) or an unrelated peptide (lanes 3 and 4). After western blotting, the filters were probed with a monoclonal antibody, SMP14 (ref. 9), against MDM2 (lanes 2 and 4) or an irrelevant antibody (lane 3). In lane 5, NIH3T3 cell extracts were probed with the MDM2 antibody. Lane 1 shows markers (200K, 97.4K, 68K and 43K).

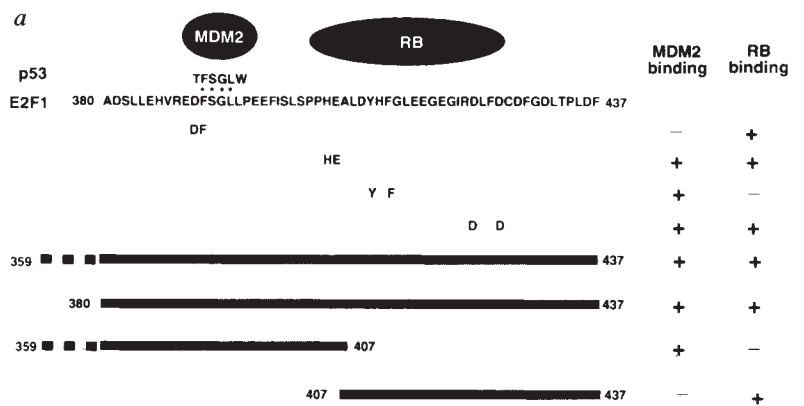
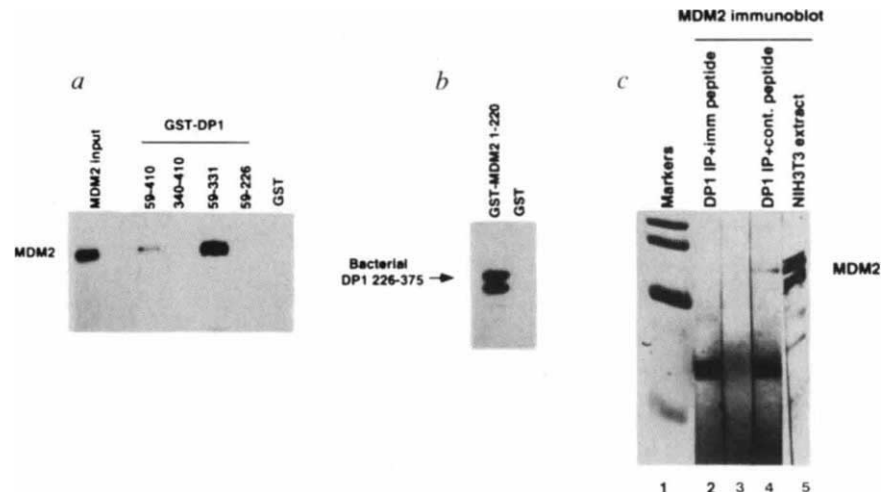


FIG. 4 MDM2 regulates the E2F1 activation domain. *a*, Deletions and mutations of GST-E2F1 are tested for binding to *in vitro* translated and radiolabelled MDM2 and the results compared with the binding of Rb¹⁰. *b*, U2OS cells were transfected with GAL4 E2F1 359 to 437 (2 µg), and *c*, GAL4 E2F1 407 to 437 (2 µg) expressed from an SV40 promoter¹⁰ in the presence or absence of CMV-MDM2. The target plasmid has GAL4 sites linked to the E1B TATA box²².

ates transcription in yeast², it is tempting to speculate that MDM2 acts as a co-activator for the E2F1 activation domain. However, we cannot exclude the possibility that MDM2 may somehow affect other protein-protein interactions with E2F1 or DP1 which ultimately influence transactivation capacity. Which-ever the mechanism, our results suggest that overexpression of MDM2 may be oncogenic, not only because it represses the activity of the p53 tumour suppressor protein, but also because it stimulates the activity of the S-phase promoting E2F1/DP1 transcription factors. □

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Interaction between the retinoblastoma protein and the oncoprotein MDM2

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INACTIVATION of tumour-suppressor genes leads to deregulated cell proliferation and is a key factor in human tumorigenesis. Both p53 and retinoblastoma genes are frequently mutated in human cancers^{1,2}, and the simultaneous inactivation of RB and p53 is frequently observed in a variety of naturally occurring human tumours³. Furthermore, three distinct DNA tumour virus groups—papovaviruses, adenoviruses and human papillomaviruses—transform cells by targeting and inactivating certain functions of both the p53 and retinoblastoma proteins^{1,2}. The cellular oncoprotein, Mdm2, binds to and downmodulates p53 function^{4–6}; its human homologue, MDM2, is amplified in certain human tumours, including sarcomas^{7–9} and gliomas¹⁰. Overproduction of Mdm2 is both tumorigenic⁴ and capable of immortalizing primary rat embryo fibroblasts¹¹. Here we show that MDM2 interacts physically and

functionally with pRB and, as with p53, inhibits pRB growth regulatory function. Therefore, both pRB and p53 can be subjected to negative regulation by the product of a single cellular proto-oncogene.

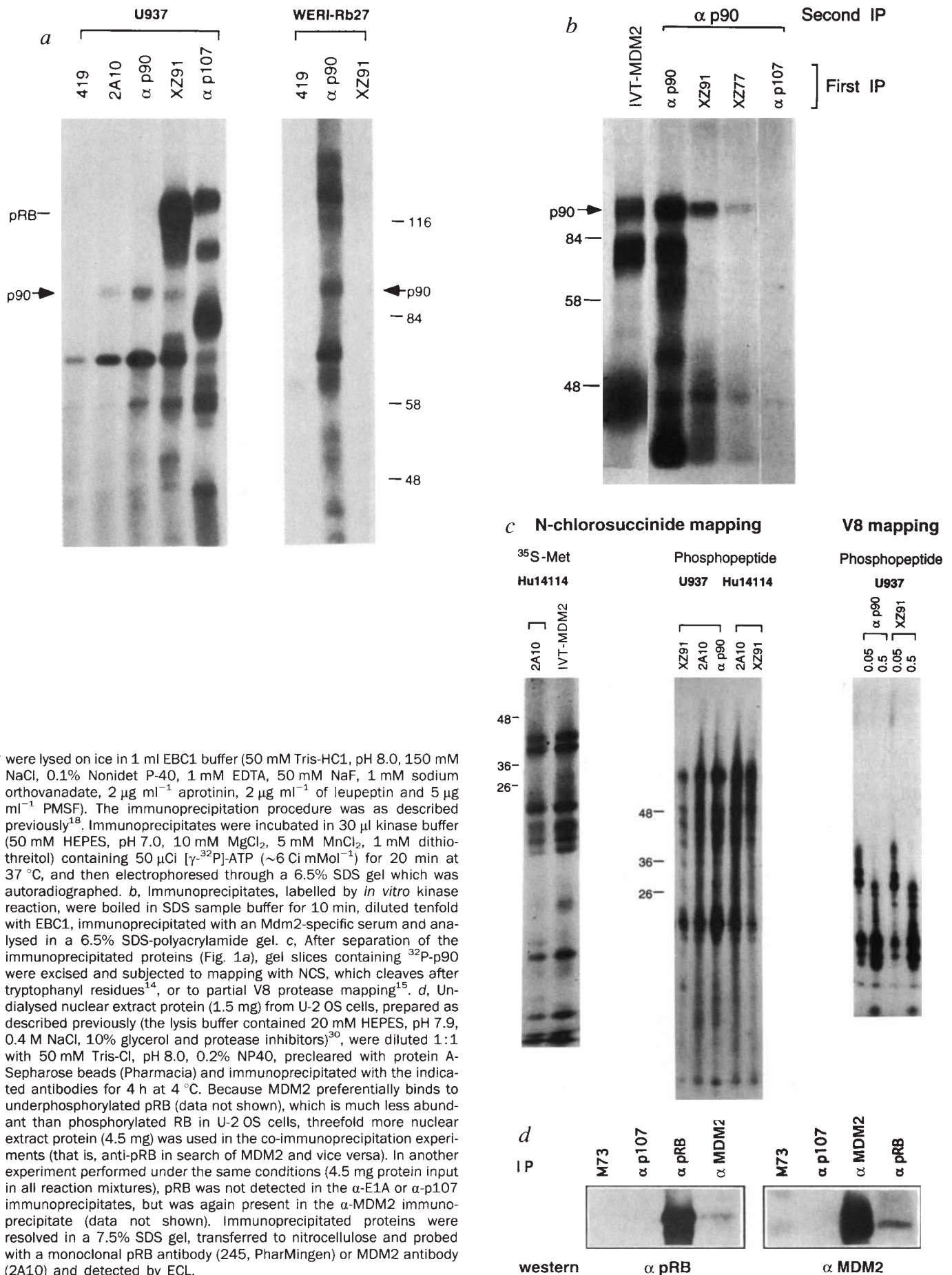
In search of cellular proteins bound to pRB, crude extracts of U937 human leukaemia cells were immunoprecipitated with a pRB-specific monoclonal antibody (XZ91)¹², and the immunoprecipitates were subjected to *in vitro* kinase labelling. A protein of *M*_r 90K (p90) was detected in these, but not in a p107 immunoprecipitate generated from the same extract (Fig. 1a). MDM2 migrated at ~90K and, when tested, p90 coprecipitated with pRB was found to comigrate with the p90 protein immunoprecipitated by either an MDM2 monoclonal antibody (2A10)¹³ or rabbit anti-Mdm2 (ap90). Neither pRB nor p90 was present in an anti-pRB immunoprecipitate of cell extracts from the retinoblastoma cell line, WERI-Rb27 (RB⁻/RB⁻) (Fig. 1a). In addition, p90 coprecipitated with pRB comigrated with MDM2 translated *in vitro*, and could be reprecipitated by serum specific for Mdm2 (Fig. 1b).

The p90 protein, which is recognized by either 2A10 or ap90, is MDM2, as the *N*-chlorosuccinimide¹⁴ (NCS)-generated peptide maps of p90, synthesized *in vivo* and immunoprecipitated with 2A10, and that of MDM2 translated *in vitro* were similar, if not identical (Fig. 1c, left panel). The NCS and V8 protease¹⁵ maps of this protein, and pRB-associated p90, both of which were labelled by *in vitro* kinase reactions, were also similar (Fig. 1c, middle and right panels). Moreover, evidence of pRB/MDM2 complexes was detected in untransfected, asynchronous U-2 OS cell lysates by co-immunoprecipitation with either pRB or MDM2 antibodies (Fig. 1d). Taken together, these results indicate that MDM2 and pRB form stable complexes *in vivo*.

The carboxy-terminal region of pRB (amino-acid residues 792 to 928) is necessary and sufficient for MDM2 binding. A series of glutathione *S*-transferase (GST)-RB fusion proteins¹⁶ were assayed for binding to *in vitro* translated MDM2. As shown in Fig. 2a, the 'large' pocket of pRB (379 to 928), a naturally occurring large pocket point mutant (C706 to F) and two truncated deletion mutants of large pocket (dl703–737 and dl738–

FIG. 1 Formation of pRB/MDM2 complexes *in vivo*. **a**, The p90 protein coprecipitated with pRB comigrates with MDM2. Cell lysates of U937 (RB^{+/+}) or WERI-Rb27 (RB^{-/-}) were immunoprecipitated by either PAb 419 (SV40 T monoclonal antibody), 2A10 (MDM2 monoclonal antibody), ap90 (anti-Mdm2 rabbit serum), XZ91 (pRB monoclonal antibody) or ap107 (a mixture of three p107 monoclonal antibodies: SD4, SD9 and SD15). Immunoprecipitates were subjected to *in vitro* kinase reactions and analysed by SDS-PAGE and autoradiography. Prestained protein marker sizes are shown on the right. **b**, Reprecipitation of pRB-coprecipitated p90 by serum specific for Mdm2. Solubilized *in vitro* kinase labelled immunoprecipitates, generated as in **a** with anti-p90, XZ91 and another RB monoclonal Ab (XZ77), were reprecipitated by a serum specific for Mdm2 (ap90) and analysed by SDS-PAGE. Lane 1 contains 2 µl of *in vitro* translated MDM2. **c**, The p90 protein coprecipitated with pRB is indistinguishable from MDM2. The left panel shows the NCS-cleavage maps of *in vitro* translated MDM2 and 2A10-immunoprecipitated p90 from [³⁵S]methionine-labelled Hu14114 (human teratocarcinoma cell line) cell lysate. The middle panel shows the NCS-cleavage maps of the *in vitro* kinase labelled products of p90 coprecipitated with pRB (XZ91) or of MDM2 precipitated by 2A10 and ap90, from U937 and Hu14114 cells, respectively. V8-partial peptide maps of *in vitro* kinase labelled p90 from U937 cells coprecipitated with pRB (XZ91) and directly precipitated by ap90 are shown on the right. The quantity of (*Staphylococcus aureus*) V8 protease used (0.05 or 0.5 µg) is indicated (top). **d**, Detection of pRB/MDM2 complexes *in vivo*. Nuclear extracts of unlabelled, untransfected U-2 OS cells were immunoprecipitated by M73 (anti-E1A monoclonal antibody), α p107 (SD4, SD9 and SD15), α pRB (XZ91) or α MDM2 (2A10 and 4B11). The resulting immunoprecipitates were resolved by SDS-PAGE and subjected to western blotting analysis using antibodies specific for pRB (245) or MDM2 (2A10) as indicated.

METHODS. **a**, U937 cells (5 × 10⁷) and WERI-Rb27 cells (1 × 10⁷) cells ►



► were lysed on ice in 1 ml EBC1 buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA, 50 mM NaF, 1 mM sodium orthovanadate, 2 $\mu\text{g ml}^{-1}$ aprotinin, 2 $\mu\text{g ml}^{-1}$ of leupeptin and 5 $\mu\text{g ml}^{-1}$ PMSF). The immunoprecipitation procedure was as described previously¹⁸. Immunoprecipitates were incubated in 30 μl kinase buffer (50 mM HEPES, pH 7.0, 10 mM MgCl_2 , 5 mM MnCl_2 , 1 mM dithiothreitol) containing 50 μCi [γ - ^{32}P]-ATP ($\sim 6 \text{ Ci mMol}^{-1}$) for 20 min at 37 $^\circ\text{C}$, and then electrophoresed through a 6.5% SDS gel which was autoradiographed. **b**, Immunoprecipitates, labelled by *in vitro* kinase reaction, were boiled in SDS sample buffer for 10 min, diluted tenfold with EBC1, immunoprecipitated with an Mdm2-specific serum and analysed in a 6.5% SDS-polyacrylamide gel. **c**, After separation of the immunoprecipitated proteins (Fig. 1a), gel slices containing ^{32}P -p90 were excised and subjected to mapping with NCS, which cleaves after tryptophanyl residues¹⁴, or to partial V8 protease mapping¹⁵. **d**, Undialysed nuclear extract protein (1.5 mg) from U-2 OS cells, prepared as described previously (the lysis buffer contained 20 mM HEPES, pH 7.9, 0.4 M NaCl, 10% glycerol and protease inhibitors³⁰), were diluted 1:1 with 50 mM Tris-Cl, pH 8.0, 0.2% NP40, precleared with protein A-Sepharose beads (Pharmacia) and immunoprecipitated with the indicated antibodies for 4 h at 4 $^\circ\text{C}$. Because MDM2 preferentially binds to underphosphorylated pRB (data not shown), which is much less abundant than phosphorylated RB in U-2 OS cells, threefold more nuclear extract protein (4.5 mg) was used in the co-immunoprecipitation experiments (that is, anti-pRB in search of MDM2 and vice versa). In another experiment performed under the same conditions (4.5 mg protein input in all reaction mixtures), pRB was not detected in the α -E1A or α -p107 immunoprecipitates, but was again present in the α -MDM2 immunoprecipitate (data not shown). Immunoprecipitated proteins were resolved in a 7.5% SDS gel, transferred to nitrocellulose and probed with a monoclonal pRB antibody (245, PharMingen) or MDM2 antibody (2A10) and detected by ECL.

775) all bound to MDM2, while intact 'small' pocket (379–792), which contains the minimum T/E1A binding domain, did not. None of these mutants can bind effectively to E2F, indicating that E2F is not required for pRB/MDM2 complex formation. However, these data do not eliminate the possibility that MDM2 binds independently to E2F.

Furthermore, the C-terminal segment of pRB (792 to 928) bound as efficiently to MDM2 as did the large pocket structure. Under the same conditions, the analogous GST-p107 large

pocket fusion protein (252 to 936) did not bind to MDM2 (data not shown). Consistent with the role of the pRB C terminus in binding to MDM2, GST-RB(379 to 928), but not GST-RB(379 to 792), bound to Mdm2 in a crude extract of 3T3-DM cells¹⁷, as assayed by western blotting (Fig. 2b).

The binding of MDM2 to the C-terminal fragment of pRB may have important implications, as this fragment, together with the small pocket, is required for the growth suppression function of pRB and for a stable interaction with E2F1 (refs 18–20), a

FIG. 2 a, *In vitro* translated MDM2 specifically binds to the C terminus of pRB. A schematic representation of results is also shown. The minimal pRB segment required for growth suppression (top), the pocket (shaded area), 706C to F mutation (vertical arrow), deletion mutations of exons 21(703 to 731) and 22(738 to 775) (black bars) are indicated. b, GST-RB(379 to 928) binds Mdm2 *in vitro*. Crude extracts of 3T3-DM cells¹⁷ were incubated with GST-RB fusion proteins as indicated. Elutes of the GST protein complexes were analysed by western blotting, using 2A10 as probe. Immunoprecipitation of the same extract was performed using 2A10, in parallel.

METHODS. a, MDM2 (15 µl) translated *in vitro* was incubated at 4 °C for 1 h with 25 µl glutathione-Sepharose beads loaded with various GST-RB fusion proteins, as described previously¹⁶. b, GST-RB fusion proteins bound to glutathione-Sepharose beads were incubated for 1 h at 4 °C with a crude extract (1 mg total protein) of 3T3-DM cells prepared by EBC lysis. Bound proteins were released, separated in a 7.5% SDS gel, and transferred to a PVDF membrane. The blot was incubated overnight with 2A10 (1:5) and processed as described previously¹⁸.

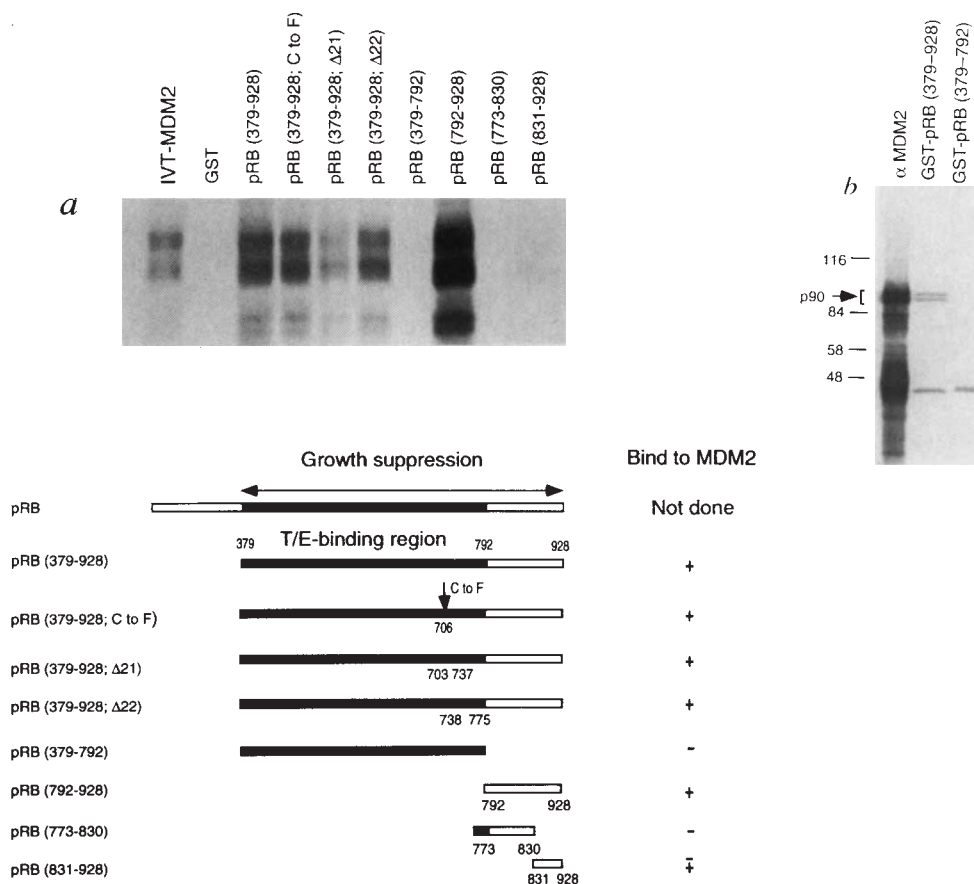
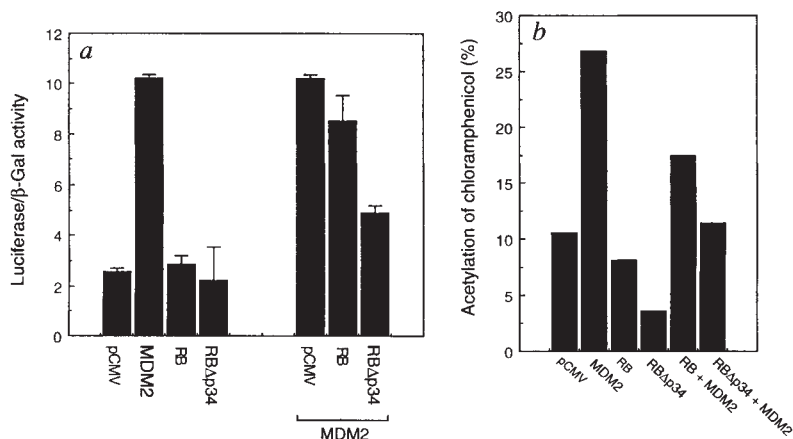


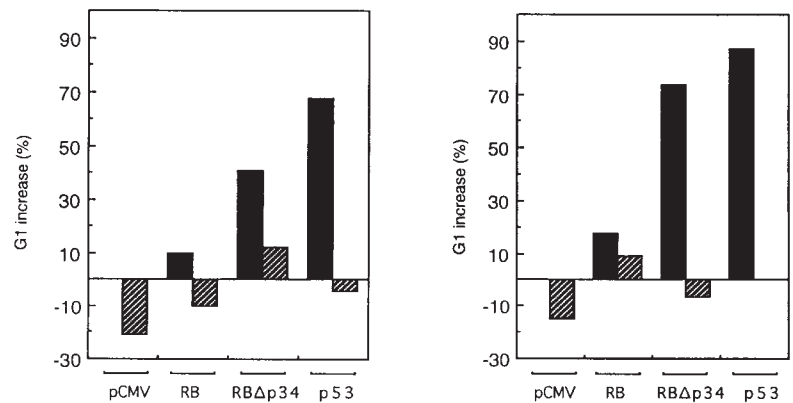
FIG. 3 a, MDM2 stimulates E2F activity, which is suppressed by cotransfection of pRBΔp34. Results are presented as a ratio of luciferase/β-galactosidase activity. b, MDM2 relieves the pRBΔp34 repression of Gal4-E2F1 transactivating function. The transactivation activity of the Gal4/E2F1 chimaeric protein was corrected for β-galactosidase activity.

METHODS. U-2 OS cells were transfected by the calcium phosphate method. a, For each 10 cm dish, 10 µg DHFR-E2F(WT)-Luciferase reporter plasmid, 1 µg pCMV-β-GAL, 15 µg pBSSK-II (Bluescript DNA) as carrier DNA, and 1 µg of each of the expression plasmids pCMV-MDM2, pCMV-pRB, pCMV-mRBΔp34HA (tagged with an influenza HA epitope)²⁴, or pCMV-p53, and 1 or 2 µg of pCMV promoter-bearing backbone plasmid were used, so that each transfection mixture contained an equal amount of pCMV plasmid. Then, 24 h after precipitate removal, cells were collected in 0.5 ml potassium phosphate buffer (0.1 M, pH 7.8) containing 1 mM DTT. Luciferase and β-galactosidase assays were performed as described previously²¹. The synthesis of MDM2, pRB and pRBΔp34 were confirmed by immunoprecipitation or western blotting (data not shown). These data were derived from multiple, independent experiments. b, For each transfection (10 cm dish), we used 4 µg reporter plasmid (3 × Gal4)BG-CAT, 1 µg pRSV-Gal4/E2F1(368 to 437)²⁵, 2 µg



pCMV-MDM2, pCMV-pRB or pCMV-mRBΔp34HA, 15 µg pBSSK-II (Bluescript), and 2 to 4 µg backbone pCMV vector, so that each transfection mixture contained the same amount of pCMV plasmid. The procedures for transfection, cell lysis, β-galactosidase and CAT assays were as described previously²¹.

FIG. 4 MDM2 overrides the *RB* Δ p34- and p53- induced G1 block in U-2 OS cells. The left and right panels show the percentage increase FITC⁺ cells in G1 from two independent experiments where U-2 OS cells were cotransfected with a CD19 expression plasmid, a pCMV plasmid lacking or encoding pRB, pRB Δ p34 or p53, in the absence (black bars) or presence of pCMV encoding MDM2 (shaded bars). **METHODS.** U-2 OS cells (~40% confluent; 10 cm plates) were cotransfected by the calcium phosphate method with 1 μ g pCMV-CD19 (encoding the surface marker CD19), 25 μ g pBSSK-II (Bluescript), 1 μ g of either pCMV-RB, pCMV-RB Δ p34 or pCMV-p53, in the absence or presence of 1 μ g pCMV-MDM2 respectively. Then, 24 to 30 h after removal of DNA precipitates, cells were collected in 1 ml PBS containing 0.1% EDTA, incubated with CD19-specific monoclonal antibody (1:500), fixed and stained with propidium iodide, and analysed by FACS as described previously²⁰. At least 25,000 cells were analysed per transfection.



transcription factor critical for G1/S transition. Unphosphorylated pRB binds E2F1/DP1 heterodimers and suppresses their transactivating function^{21,22}. There is a strong correlation between pRB/E2F complex formation and pRB blockade of the G1/S transition¹⁸⁻²⁰.

To discover whether binding of MDM2 to pRB perturbs the regulation of E2F activity by pRB, an *MDM2* expression plasmid and an E2F reporter (DHFR-luciferase²³) were cotransfected into U-2 OS, an *RB*⁺/*p53*⁺ line of human osteogenic sarcoma cells. This resulted in significant stimulation of endogenous E2F transactivating function (Fig. 3a, left), consistent with MDM2 forming complexes with pRB and relieving pRB suppression of E2F transactivating function. By contrast, a DHFR-luciferase reporter containing a mutant E2F site was much less active in U-2 OS cells than the wild type, and overproduction of MDM2 resulted in significantly less stimulation of this reporter (data not shown). The MDM2 dependent stimulation of the wild-type E2F promoter was significantly suppressed by coexpression of pRB Δ p34 (Fig. 3a, right), a superactive pRB mutant containing mis-sense mutations at 8 potential cdk phosphorylation sites and, therefore, resistant to *in vivo* phosphorylation²⁴. We found that pRB Δ p34 contains an intact, functional pocket and C-terminal unit and, when overproduced, it can bind E2F and suppress the growth of certain *RB*⁺ cells (W.R.S., W. Kaelin and D.M.L., unpublished results).

MDM2 not only stimulated endogenous E2F activity, but also enhanced the transactivating function of a Gal4-E2F1 fusion protein²⁵ in pRB-containing cells, and relieved the pRB Δ p34-dependent repression of Gal4-E2F1 transactivation activity²⁵ (Fig. 3b). These observations further suggest that MDM2 can perturb pRB suppression of E2F function. Stimulation of E2F by MDM2 may be a pRB-independent phenomenon, for example, arising by means of an interaction between MDM2 and a member of the E2F family. However, when tested, MDM2 transfection into *RB*^{-/-} cells (Saos-2) did not appear to stimulate E2F reporter activity (data not shown).

Wild-type pRB, when overproduced in U-2 OS, is efficiently phosphorylated and does not lead to cell cycle arrest (C. Fuchs, J. DeCaprio, X. Qin, W. Kaelin and D.M.L., unpublished observations). By contrast, transfection of either *RB* Δ p34 or a wild-type *p53* expression plasmid into U-2 OS led to G1 arrest (Fig. 4). Cotransfection of *MDM2* significantly inhibited *RB* Δ p34 (Fig. 4) and p53-mediated G1 arrest activities (Fig. 4 left). This effect of MDM2 was also observed in U-2 OS cells cotransfected with both p53 and *RB* Δ p34 expression plasmids (data not shown). Therefore MDM2 neutralized the G1 blocking effects of two known tumour and growth suppressors. In keeping with this conclusion, transfection of *MDM2* alone stimulated G1 exit of these *RB*⁺/*p53*⁺ cells (Fig. 4).

MDM2 also suppressed the large cell phenotype induced by pRB in Saos-2 cells^{20,26} (data not shown) which lack functional

p53, suggesting that MDM2 can perturb pRB function in a p53-independent manner.

Both pRB and p53 are targeted by certain DNA tumour viruses, and both are frequently inactivated in certain naturally occurring human tumour cells, including sarcomas, small cell lung, oesophageal and cervical carcinomas (ref. 3 and references therein), implying a cooperative and/or overlapping functional relationship between these two proteins. One possible basis for the linkage of dual p53 and pRB inactivation is that loss of pRB function can lead to p53-dependent apoptosis, whereas loss of both functions can result in cell survival²⁷⁻²⁹.

It is also possible that both proteins operate in an overlapping manner in blocking the G1/S transition, and that downregulating only one of them is not sufficient for cell cycle progression in certain settings. The results presented here indicate that p53 and pRB are both susceptible to the inhibitory effects of a single oncoprotein, supporting the idea that the growth-suppressing functions of these two proteins operate in parallel. In that case, MDM2 is a cellular protein which, like papovaviral T antigen, can inhibit the action of both tumour-suppressor gene products. That both cells and viruses have evolved analogous mechanisms of pRB and p53 perturbation raises the interesting question of whether these two proteins communicate with one another during the normal performance of their growth regulatory functions. □

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Non-genetic propagation of strain-specific properties of scrapie prion protein

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THE infectious agents causing scrapie and other transmissible spongiform encephalopathies have been postulated to consist solely of the protease-resistant form of prion protein (PrP^{Sc})^{1–6}. One unprecedented requirement of the protein-only model is that the 'inheritance' of pathogen strain differences must be mediated by stable variations in PrP^{Sc} structure^{2,7,8}, rather than mutations in an agent-specific nucleic acid⁹. Strain differences in PrP^{Sc} structure have been described for the hyper (HY) and drowsy (DY) strains of hamster transmissible mink encephalopathy (TME)^{7,8}, a scrapie-like disease originating in mink. Although HY and DY PrP^{Sc} are both post-translationally derived from the precursor prion protein (PrP^C) they are cleaved at different amino-terminal sites by proteinase K (ref. 8). Here we investigate whether this strain-specific property of PrP^{Sc} is transmitted to PrP^C during formation of new PrP^{Sc}. PrP^{Sc} from the HY and DY TME strains converted the protease-sensitive PrP^C into two distinct sets of protease-resistant PrP products in a cell-free system. These data provide evidence that self-propagation of PrP^{Sc} polymers with distinct three-dimensional structures could be the molecular basis of scrapie strains.

To investigate the mechanism of scrapie strain propagation, we tested whether the biochemical differences between HY and DY PrP^{Sc} (Fig. 1a; refs 7, 8) can be transmitted to PrP^C. This was performed using a cell-free system⁶ in which PrP^{Sc} induces the conversion of PrP^C to a proteinase K (PK) resistant form. The species specificity of the cell-free conversion system correlates with *in vivo* barriers to interspecies transmission of scrapie¹⁰. The major constituents of the cell-free conversion assay are recombinant hamster [³⁵S]-methionine-labelled PrP^C (³⁵S-PrP^C) isolated from uninfected tissue culture cells and PrP^{Sc} derived from TME-infected hamster brain. After incubation, the mixture was digested with PK and analysed by SDS-PAGE and fluorography. This procedure detected PK-resistant ³⁵S-PrP products derived only from PK-sensitive ³⁵S-PrP^C precursors. Figure 1b shows that hamster brain PrP^{Sc} from the HY and DY TME strains converted the same ³⁵S-PrP^C precursors (consisting of a major unglycosylated isoform of relative molecular mass 24,000 (*M*_r 24K) and a minor glycosylated isoform (*M*_r 26K) into distinct sets of PK-resistant ³⁵S-PrP products. The hamster ³⁵S-PrP^C precursors derived from the cell line (Fig. 1b, lane 5)

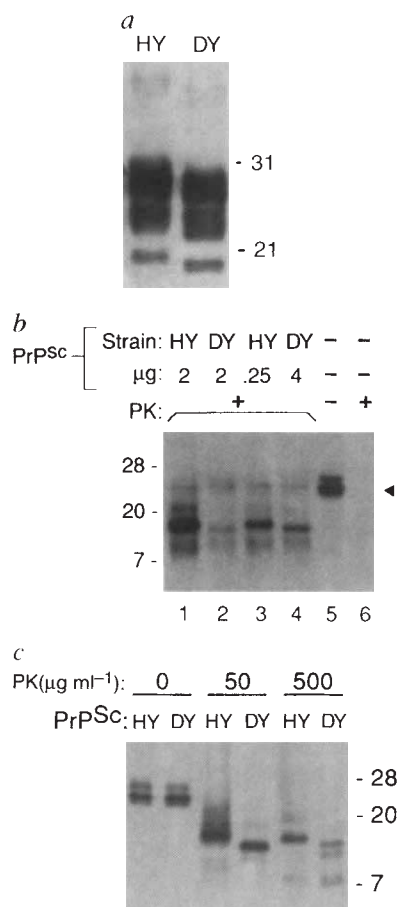


FIG. 1 Strain-specific properties of HY and DY PrP^{Sc} and cell-free conversion of PrP^C to PK-resistant PrP. a, Immunoblot of purified brain-derived PrP^{Sc} digested with PK from HY and DY TME-infected hamsters. HY and DY PrP^{Sc} are both post-translationally derived from hamster PrP^C. *In vitro* they are cleaved at different N-terminal sites by proteinase K, resulting in PK-resistant PrP^{Sc} polypeptides that differ in *M*_r by 1K^{7,8}. Note the faster migration of DY PrP^{Sc} polypeptide bands compared with HY PrP^{Sc}. b, Cell-free conversion reactions containing either 2 µg HY or DY PrP^{Sc} (lanes 1 and 2), or 0.25 µg HY PrP^{Sc} (lane 3) or 4 µg DY PrP^{Sc} (lane 4) combined with ³⁵S-PrP^C. Lane 5 contains ³⁵S-PrP^C without PrP^{Sc}. Lane 6 contains ³⁵S-PrP^C without PrP^{Sc} in the presence of PK. c, Cell-free conversion products digested with increasing concentrations of PK. The major recombinant ³⁵S-PrP^C has *M*_r 24K (indicated by the arrowhead), which is smaller than the hamster brain-derived PrP^C (*M*_r 33K to 35K) because it lacks N-linked glycans and a glycosylphosphatidylinositol anchor⁶. These differences between the sources of PrP^C also can explain why the banding pattern of the PK-resistant ³⁵S-PrP in SDS-PAGE was different from the three PK-resistant PrP^{Sc} bands derived from hamster brain PrP^C, but for both sources there was a reduction in *M*_r of ~7K after PK digestion. In some experiments, residual levels of the ³⁵S-PrP^C of *M*_r 24K were present after PK digestion.

METHODS. a, The HY and DY strains of agent were isolated in Syrian hamsters after serial passage of hamster brain inoculated with the transmissible mink encephalopathy agent¹². These strains produce distinct incubation periods, clinical symptoms, neuropathological lesions, and distributions of brain PrP^{Sc} (refs 8, 12). Purification of PrP^{Sc} from hamster brain was described previously⁸. b, Cell-free reactions consisted of cell-culture-derived hamster [³⁵S]-methionine labelled recombinant PrP^C (60,000 c.p.m.) in 3.0 M GdnHCl combined with hamster brain HY or DY PrP^{Sc} (not digested with proteases and >80% PrP purity by SDS-PAGE) also in 3.0 M GdnHCl followed by dilution to 0.75 M GdnHCl in 50 mM Tris-HCl (pH 7.4), as described previously⁶. After incubation for 2 days at 37 °C, the mixture was diluted to 0.4 M GdnHCl and either digested with PK (+) at 20 µg ml⁻¹ or untreated (–) for 1 h at 37 °C. Conversion reaction ³⁵S-PrP products were analysed by 15% SDS-PAGE and fluorography. c, Cell-free conversion reactions were performed as above except that 1.5 mM CPC was included as described in Fig. 2. In addition, either 50 µg ml⁻¹ or 500 µg ml⁻¹ PK was used in the protease digestion step. *M*_r markers are indicated.

were reduced in M_r after PK digestion to major polypeptides of either 16K or 17K when ^{35}S -PrP^C was incubated with DY or HY PrP^{Sc}, respectively (Fig. 1b, lanes 1–4). The strain-specific PK-resistant ^{35}S -PrP products were not eliminated after digestion with higher concentrations of PK (Fig. 1c). These major PK-resistant ^{35}S -PrP products were also generated in the presence of only unglycosylated 24K ^{35}S -PrP^C (purified from tunicamycin-treated cells), confirming that these products were derived from the same polypeptide precursor (data not shown). ^{35}S -PrP^C was completely degraded by PK in the absence of PrP^{Sc} (Fig. 1b, lane 6) indicating that a PrP^{Sc} template is required for the conversion to PK-resistant forms. These results indicated that HY and DY PrP^{Sc} have distinct PrP-converting activities. The 1K difference in M_r between the predominant PK-resistant PrP products from each strain was similar to the difference between HY and DY brain PrP^{Sc} after digestion with PK (Fig.

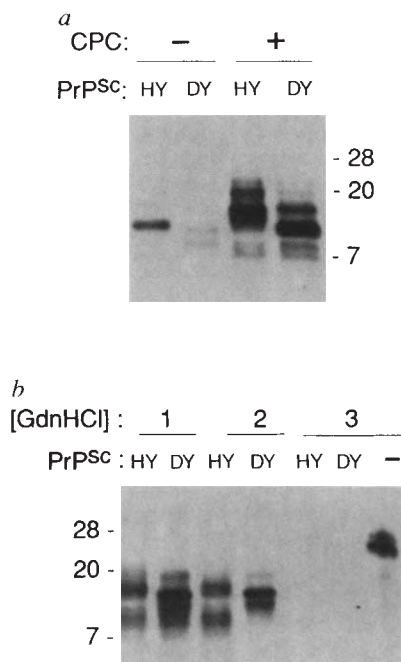


FIG. 2 The effects of cetylpyridinium chloride (CPC) and guanidine hydrochloride (GdnHCl) on the strain-specific PrP conversion reactions. **a**, The conversion reaction was as described in Fig. 1b except in the absence (-) or presence (+) of 1.5 mM CPC. In the presence of CPC, the major PK-resistant ^{35}S -PrP conversion products had M_r 18K to 22K after incubation with HY PrP^{Sc}. In the DY PrP^{Sc} reaction, the addition of CPC did not change the migration of the major 16K conversion product, although different minor conversion products were formed. **b**, The cell-free conversions were performed in varying concentrations of GdnHCl to determine the effect of denaturing conditions on the strain-specific conversion assay. The absence of PK-resistant PrP products after incubation in 3.0 M GdnHCl was probably due to irreversible denaturation of PrP^{Sc} rather than ^{35}S -PrP^C; pretreatment of ^{35}S -PrP^C with 7.5 M GdnHCl does not inhibit its conversion to PK-resistant PrP, but hamster PrP^{Sc} loses its ability to mediate conversion when pretreated with >3 M GdnHCl^{6,10}. Alternatively, 3.0 M GdnHCl could block PrP intermolecular interactions which are important for the conversion of ^{35}S -PrP^C into PK-resistant PrP.

METHODS. The cell-free conversion reactions were as described in Fig. 1 except in the presence of 1.5 mM CPC (**a**) or 1.0 mM CPC (**b**). **b**, At 1.0 mM CPC the PK-resistant ^{35}S -PrP conversion products had M_r values slightly different from those obtained at 1.5 mM CPC (Fig. 2a). The cell-free conversion reactions were as described above except that the final GdnHCl concentration in the reaction was adjusted to either 1 M, 2 M or 3 M immediately before digestion with PK the reactions were diluted to 0.4 M GdnHCl. ^{35}S -PrP^C in the absence (-) of PrP^{Sc} was not digested with PK. The arrowhead marks the M_r 24K ^{35}S -PrP^C precursor.

1a). Because the strain-specific ^{35}S -PrP products produced in these cell-free reactions were derived from the same unglycosylated ^{35}S -PrP^C precursor, differences in polypeptide three-dimensional structure probably account for the distinct PK digestion patterns.

The conversion of ^{35}S -PrP^C to PK-resistant PrP was more efficient in the presence of HY PrP^{Sc} than DY PrP^{Sc} (Fig. 1b, lanes 1 and 2). Increasing the amount of DY PrP^{Sc} in the conversion reaction by 16-fold relative to HY PrP^{Sc} resulted in approximately equal ^{35}S -PrP band intensities between the strains (Fig. 1b, lanes 3 and 4). The quaternary ammonium detergent cetylpyridinium chloride (CPC) was found to enhance the efficiency of the reaction as much as 3.5-fold with HY PrP^{Sc} and 14-fold with DY PrP^{Sc} (Fig. 2a). The pattern of the PK-resistant ^{35}S -PrP conversion products was slightly altered in the presence of CPC, but there were still M_r differences between the major PK-resistant ^{35}S -PrP products from the two strains. Similarly, increasing the concentration of denaturant up to 2.0 M GdnHCl (from 0.75 M in Figs 1 and 2a) in the cell-free reaction maintained the strain-specific conversion patterns (Fig. 2b), but when the conversion reaction was performed in 3.0 M GdnHCl, all of the ^{35}S -PrP was degraded by PK. In these reactions, CPC and GdnHCl influenced both the extent of conversion and the nature of the ^{35}S -PrP conversion products but did not eliminate the strain specificity of the conversion reactions. This provided additional evidence that the strain specificity of the conversion reactions was maintained under a variety of conditions and, therefore, was due to stable differences between HY and DY PrP^{Sc}.

Proteinase K digestion of HY and DY PrP^{Sc} before they were used in the cell-free conversion did not alter their ability to convert ^{35}S -PrP^C into strain-specific PK-resistant ^{35}S -PrP products (Fig. 3). This pretreatment helped eliminate minor protein contaminants from the brain-derived PrP^{Sc} preparations, and cleaved HY and DY PrP^{Sc} at distinct N-terminal sites⁸. The ability of PK-digested HY and DY PrP^{Sc} preparations to work effectively in the cell-free conversion assay suggests that direct interactions between PrP^{Sc} and PrP^C are sufficient to mediate the strain-specific conversion reactions, and make it less likely that other proteins are required. If additional factors are involved in the cell-free conversion reaction then they must co-purify with PrP^{Sc} and be PK resistant.

Our study demonstrates that PrP^{Sc} from two strains of hamster TME can convert the same unglycosylated PrP^C precursor into two distinct sets of PK-resistant PrP products. Although the precise mechanism of this conversion is not known, the formation of PK-resistant ^{35}S -PrP can occur in a cell-free environment and in the absence of nucleic acid replication. It is important to determine whether the conversion of ^{35}S -PrP^C into PK-resistant ^{35}S -PrP results in the formation of new TME infec-

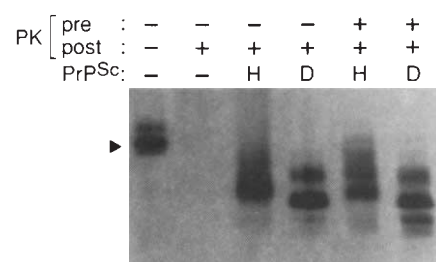


FIG. 3 Effect of PK pretreatment of PrP^{Sc} on the strain-specific cell-free conversion reaction. HY and DY PrP^{Sc} were digested (+) with PK (pre) to generate preparations highly enriched for PrP^{Sc} (>95% by SDS-PAGE), as described previously⁸. Predigested and undigested (-) PrP^{Sc} were used in the cell-free conversion assay followed by the PK digestion step (post), as described in Fig. 2a. The arrowhead marks the M_r 24K ^{35}S -PrP^C precursor.

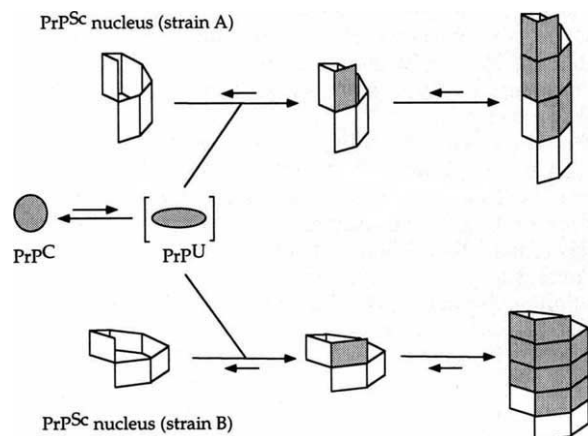


FIG. 4 Nucleation-dependent protein polymerization model for strain differences in scrapie and other transmissible spongiform encephalopathies. In this model, monomeric PrP^C exists primarily in a soluble conformation (shaded circle). Under certain cellular conditions, an unfolded conformation of PrP (PrP^U, shaded oval) and/or an insoluble conformation (shaded rectangle) may be in rapid equilibrium with PrP^C but are sparsely populated at equilibrium. In the absence of PrP^{Sc}, the concentration of the insoluble conformer would be too low to allow nucleus formation and polymerization. However, in the presence of a PrP^{Sc} nucleus, or seed, the insoluble PrP conformer can be stabilized by intermolecular interactions with PrP^{Sc}, leading to additional growth of the PrP^{Sc} polymer. Alternatively, the PrP^{Sc} nucleus could directly catalyse a conformational change in the soluble PrP^C monomer on addition to the PrP^{Sc} polymer¹³. According to this model, strain differences would be due to PrP^{Sc} polymers that have distinct packing arrangements and/or conformations. The same monomeric PrP conformer could be packed into differently arranged PrP^{Sc} polymers (as shown here) or distinct PrP conformers could be incorporated into different PrP^{Sc} polymers. PK digestion of different PrP^{Sc} polymers could generate distinct cleavage products even if they are derived from the same PrP^C precursor. Although many proteins self-assemble into polymers by nucleation-dependent polymerization^{4,14}, this would be the first example of an infectious pathogen using only this mechanism for replication and strain variation.

tivity. However, in the current conversion system there is far more brain PrP^{Sc} than ³⁵S-PrP^C (ref. 6), making detection of new TME infectivity very difficult.

The existence of scrapie strains has been difficult to explain by the protein-only hypothesis^{9,11}. A simple, biochemically preceded mechanism for PrP^{Sc} formation based on nucleation-dependent protein polymerization has been proposed^{3,4,6,10}. Based on this model, scrapie strain differences can now be reconciled with the protein-only hypothesis (Fig. 4). In this scenario, scrapie strains are alternative conformations or packing arrangements of PrP^{Sc} polymers, analogous to the alternative crystal forms which have been observed for many proteins. Our present data indicating that the pre-existing PrP^{Sc} can determine the structure of the newly formed PrP^{Sc} is consistent with this model. These results demonstrate that the chemical conditions of the reaction can strongly affect the efficiency of the conversion. Similarly, diversity of the chemical or physiological conditions within the brain could determine the strain-specific tissue distribution and rate of formation of HY and DY PrP^{Sc}. These differences in PrP^{Sc} formation can determine the strain-specific clinical and pathological features of scrapie diseases. □

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Crystal structure of isopenicillin N synthase is the first from a new structural family of enzymes

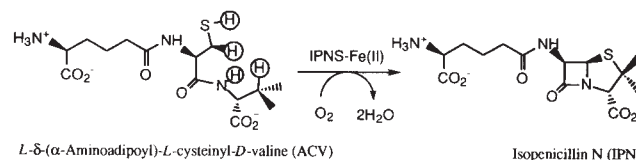
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PENICILLIN antibiotics are all produced from fermentation-derived penicillins because their chemical synthesis is not commercially viable. The key step in penicillin biosynthesis, in which both the β -lactam and thiazolidine rings of the nucleus are created, is mediated by isopenicillin N synthase (IPNS), which binds ferrous iron and uses dioxygen as a cosubstrate. In a unique enzymatic step, with no chemical precedent, IPNS catalyses the transfer of four hydrogen atoms from its tripeptide substrate to dioxygen forming, in a single reaction, the complete bicyclic nucleus of the penicillins. We now report the structure of IPNS complexed with manganese, which reveals the active site is unusually buried within a 'jelly-roll' motif and lined by hydrophobic residues, and suggest how this structure permits the process of penicillin formation. Sequence analyses indicate IPNS, 1-aminocyclopropane-1-carboxylic acid oxidase and many of the 2-oxo-acid-dependent oxygenases contain a conserved jelly-roll motif, forming a new structural family of enzymes.

The story of penicillin is one replete with surprises, from its serendipitous discovery², its unusual chemical structure and efficacy as an antibiotic^{3,4}, and more recently that its biosynthesis is dependent upon iron and dioxygen⁵. The key step in penicillin biosynthesis is the transformation of the linear tripeptide L- δ -(α -aminoadipoyl)-L-cysteinyl-D-valine (ACV) into isopenicillin N (IPN) by the loss of four hydrogen atoms in a desaturative ring closure with concomitant reduction of dioxygen to water.



Thus, the full four-electron oxidizing power of dioxygen is used in this IPNS-catalysed desaturative step, unlike other non-haem ferrous iron-dependent oxygenases and oxidases which require electron donors or oxidizable cosubstrates⁶.

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TABLE 1 Data collection and phasing statistics

Compound	Resolution (Å)	Number of observations	Unique reflections	Completeness (%)	R_{merge}^* (%)	Isomorphous difference† (%)	Number of sites	Phasing power‡ (acentric/centric)
Native	2.5	136,252	36,720	98.1	10.9			
EMP§	3.0	79,890	21,416	97.9	14.2	20.6	4	1.43/1.13
Baker's di-mercurial	3.5	44,943	13,961	94.4	12.5	30.2	4	1.56/1.22

Recombinant IPNS from *Aspergillus nidulans*⁸ was crystallized in the presence of 2.5 mM MnCl_2 (ref. 9). The crystals (0.5–1.0 mm overall dimensions) belong to space group $P2_12_12_1$ with unit cell dimensions of $a = 59.2$ Å, $b = 127.0$ Å, $c = 139.6$ Å. The asymmetric unit contains a dimer, and the solvent content of the crystals is 60%. The structure of the enzyme was solved by the method of multiple isomorphous replacement (MIR) from two heavy-atom derivatives. The derivatives were prepared by soaking native crystals at 20–21 °C in 0.5 mM solutions of the respective heavy-atom reagents for 16–20 h. High-resolution native data were collected using 0.893 Å radiation at beamline 4 of the ESRF (30 cm MAR research image plate detector). All other data were collected in-house on an 18 cm MAR Research image plate detector using CuK_α radiation from a Rigaku rotating anode generator operating at 60 kV, 70 mA. The data were processed with the DENZO program¹⁹ and the MIR phases were improved by non-crystallographic symmetry averaging, solvent flattening and histogram matching using the CCP4 Suite of Programs (Program DM)²⁰. The two derivatives had two sites in common. Electron density maps were interpreted with the program O²¹. Skeletonized maps²² were calculated for chain tracing. Intermediate models of the enzyme were refined by simulated annealing using the program X-PLOR²³ with non-crystallographic symmetry restraints imposed. During 7 cycles of refinement and rebuilding, 328 residues (Gly 2 and Ser 3 were not visible in each subunit) could be fitted to the density in both molecules of the asymmetric unit. In the final cycle, the positions of all the atoms in the asymmetric unit including 194 water molecules, two active-site-bound manganese ions, and two additional manganese ions (bound to Glu 81 and His 82 in each subunit) were refined without non-crystallographic symmetry restraints. At present, the crystallographic R factor (defined as $R_{\text{cryst}} = \sum |F_{\text{obs}}| - |F_{\text{calc}}| / \sum |F_{\text{obs}}| \times 100$) in the resolution range of 8–2.5 Å is 22.0% for all observations and 21.5% using 33,229 reflections with $F_{\text{obs}} > 2\sigma(F_{\text{obs}})$. The free R value²⁴ is 26.5% based on 1,449 randomly selected reflections (4% of the total) and 26.4% for 1,425 reflections with $F_{\text{obs}} > 2\sigma(F_{\text{obs}})$. The r.m.s. deviation of the bond lengths, bond angles and torsion angles from standard values is 0.01 Å, 2° and 24°, respectively. The mean coordinate error is 0.45 Å based on the SIGMAA method²⁵. The r.m.s. difference between subunits is 0.32 Å for C_α atoms and 0.72 Å for all atoms. Gln 330, which is coordinated to the active-site metal, is the only Ramachandran outlier in each chain (chain A, $\phi = 120^\circ$, $\psi = 100^\circ$; chain B, $\phi = 112^\circ$, $\psi = 90^\circ$).

* $R_{\text{merge}} = \sum_j \sum_h |I_{h,j} - \langle I_h \rangle| / \sum_j \sum_h \langle I_h \rangle \times 100$, where $I_{h,j}$ is the intensity of an individual measurement, and $\langle I_h \rangle$ is the mean intensity of that reflection.

† Per cent isomorphous difference is calculated using the expression $\sum |F_{\text{PH}}| - |F_P| / \sum |F_P| \times 100$, where F_P and F_{PH} refer to the native and the derivative structure factor.

‡ The phasing power of a derivative is defined as the ratio of the amplitude of the r.m.s. heavy-atom scattering factor to the r.m.s. lack of closure.

§ Ethyl mercury phosphate.

|| 1,4-Diacetoxymercuro-2,3-dimethoxybutane.

Initially IPNS from *Cephalosporium acremonium* was crystallized, but the crystals obtained were of insufficient quality for structure determination⁷. Crystals of a recombinant *Aspergillus nidulans* IPNS⁸ complexed with manganese⁹ were then obtained and were used to determine the structure of IPNS at a resolution of 2.5 Å (Table 1, Fig. 1). These crystals contain manganese instead of iron at the active site and are relatively stable under aerobic conditions. The secondary structure of the enzyme consists of 10 helices and 16 β -strands (Fig. 1). Eight of the β -strands ($\beta 5$, $\beta 8$ –14, Figs 1 and 2) fold to give a jelly-roll motif¹⁰. The jelly-roll motif is common among viral capsid proteins¹⁰ and has been identified in enzymes^{11,12}. Unlike other known jelly-roll proteins^{10,12}, in IPNS the jelly roll is not a completely closed structural unit and the active site is buried within the β -barrel. The side of the jelly roll consisting of β -strands 12, 9, 14 and 5 is extended at both ends by strands 1, 2, and 4, 5, respectively, to form a larger sheet (Fig. 1). The continuation of the β -sheet from $\beta 5$ allows the C-terminal tail (324–331) extending from the final α -helix ($\alpha 10$) to enter between the two faces of the jelly-roll, allowing Gln 330 to ligate to the metal. This glutamine is present in all independently determined IPNS sequences. The other side of the jelly roll consists of strands 8, 13, 10 and 11. Strands 6 and 7 form a hairpin loop on the surface. The longest α -helix ($\alpha 6$) straddles β -strands 4, 5, 9, 12 and 14 on the outside of the jelly roll and is linked by a sharp turn containing the conserved Gly 165 to another strand of chain (166–178) containing $\alpha 7$ (Fig. 1b).

The active-site structure (Fig. 1d) reveals the manganese ion, substituting for the ferrous ion at the metal binding site. It is attached by four protein ligands (His 214, Asp 216, His 270, Gln 330) and bears two water molecules, the latter occupying coordination sites directed into a hydrophobic cavity within the protein. It is likely that ACV and dioxygen bind to the coordination sites occupied by the water molecules and Gln 330 (Fig. 3). Such a structural characteristic (an iron-binding site within an unreactive hydrophobic substrate binding cavity) is probably a requirement for this class of enzyme, as it results in the isolation of the reactive complex and subsequent intermediates (Fig. 3)

from the external environment. Thus, the reaction can be channelled along a single path, avoiding the many side reactions potentially open to the highly reactive species resulting from the reduction of dioxygen at the metal. The role of enzymes in such processes has been characterized as negative catalysis¹³.

Previous sequence comparisons between IPNS, 1-aminocyclopropane-1-carboxylic acid oxidase and some 2-oxo-acid-dependent oxygenases have identified homologous regions, including two containing the active site histidines of IPNS (His 214 and His 270)¹⁴. For some other 2-oxoglutarate-dependent oxygenases convincing sequence alignments have not been achieved^{14,15}. In the light of the structure of IPNS, sequence comparison^{16,17} of IPNS with 1-aminocyclopropane-1-carboxylic acid oxidase and related 2-oxo-acid-dependent oxygenases (Figs 1 and 2) leads us to propose that many of them use the same basic structural platform and have thus evolved through a divergent process. In particular the jelly-roll core and the longest α -helix ($\alpha 6$) in IPNS are highly conserved. The presence of the $\alpha 6$ helix may be concerned with stabilization of the distorted jelly-roll motif (Fig. 1). These comparisons also indicate that the active site iron is likely to be coordinated by the side chains of the conserved aspartyl (Asp 216) and histidyl (His 214, His 270) residues for the other members of the family. The conservation of structural and active-site motifs throughout a number of these enzymes suggests that they operate through closely related mechanisms. Gln 330 which is also ligated to the manganese bound to the active site in the IPNS structure and is presumably involved in catalysis is conserved throughout all reported IPNS sequences, but is not conserved through other members of the family. It may be that this difference in coordination chemistry between IPNS and the other members reflects differences in their mechanism and substrate specificity, in particular the fact that IPNS does not use a 2-oxo-acid cosubstrate.

IPNS, 1-aminocyclopropane-1-carboxylic acid oxidase and the related 2-oxo-acid-dependent oxygenases constitute a new structural family which catalyses a wide range of oxidative chemistry, including hydroxylation, desaturation and desaturative

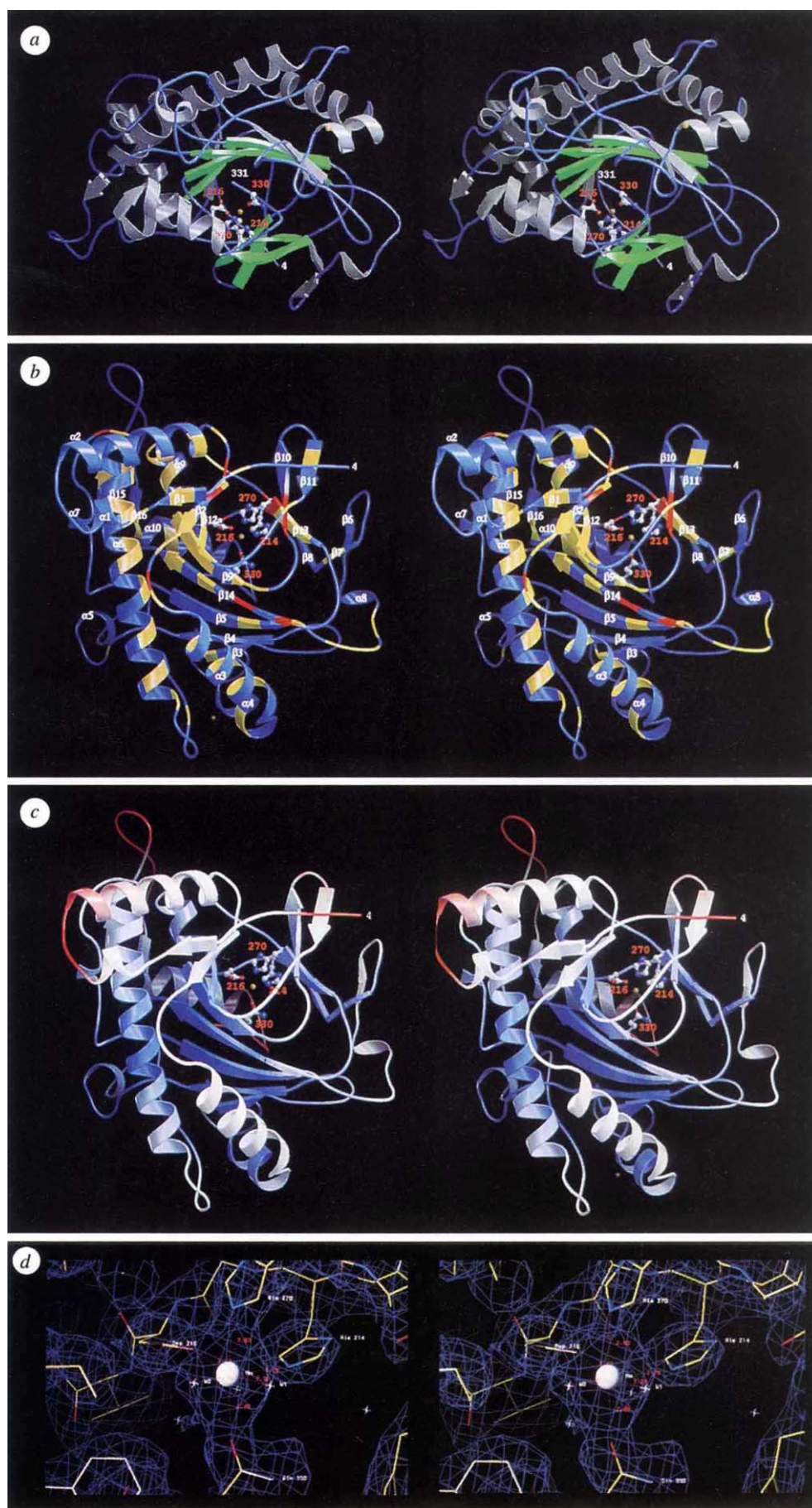


FIG. 1 *a-d*, The structure of IPNS. *a*, The three-dimensional structure of isopenicillin *N* synthase showing the conserved jelly-roll motif in green. The structure is viewed from the entrance to the active site. The active-site bound metal and its ligands are buried within the jelly roll. A second and weaker metal-binding site is located on the surface of the molecule in a non-conserved area with Glu 81 and His 82 as protein ligands. The secondary structure elements of the molecule are shown in grey except for the conserved β -strands of the jelly-roll motif (green). Residue 331 is the C-terminal threonine, and residue 4 is the first N-terminal residue that could be localized in the structure. *b*, Distribution of conserved residues in IPNS and related enzymes. The molecule is viewed from the back of the active site. Results from the sequence comparisons of Fig. 2 are mapped on the three-dimensional image of the molecule. The colouring scheme follows the convention of Fig. 2: sequence identities are shown in red and residues with similar physical-chemical properties in yellow. *c*, Structure coloured according to crystallographic temperature factors. Blue shows the smallest values and red the highest. Comparison with *c* shows that the distribution of low *B* factor residues in the structure largely follows the distribution of conserved residues in the family. *d*, Stereo-view of the active site region of IPNS. The figure is taken from the same orientation as *b* and *c*. Electron density was contoured at 1.4σ , where σ represents the r.m.s. electron density for the unit cell. The structure shows a distorted octahedral geometry around the manganese, with the ligands formed from two water molecules and the side chains of His 214, His 270, Asp 216 and Gln 330 (Fig. 2). The latter was unanticipated. An analysis of the protein geometry around the metal site indicates strain in the neighbouring polypeptide structure. The carboxylate of Thr 331 forms a H bond with Arg 87. The metal ligand Gln 330 is the only Ramachandran outlier. The coordination chemistry of IPNS is to our knowledge unique. Probably the most related coordination chemistry is found in the iron and manganese superoxide dismutases, where the metal ligands are provided by the side chains of an aspartyl and three histidyl residues, with a nearby glutamyl residue which is not directly ligated to the metal. *a*, *b* and *c* were produced using MOLSCRIPT²⁶, *d* using O²¹.

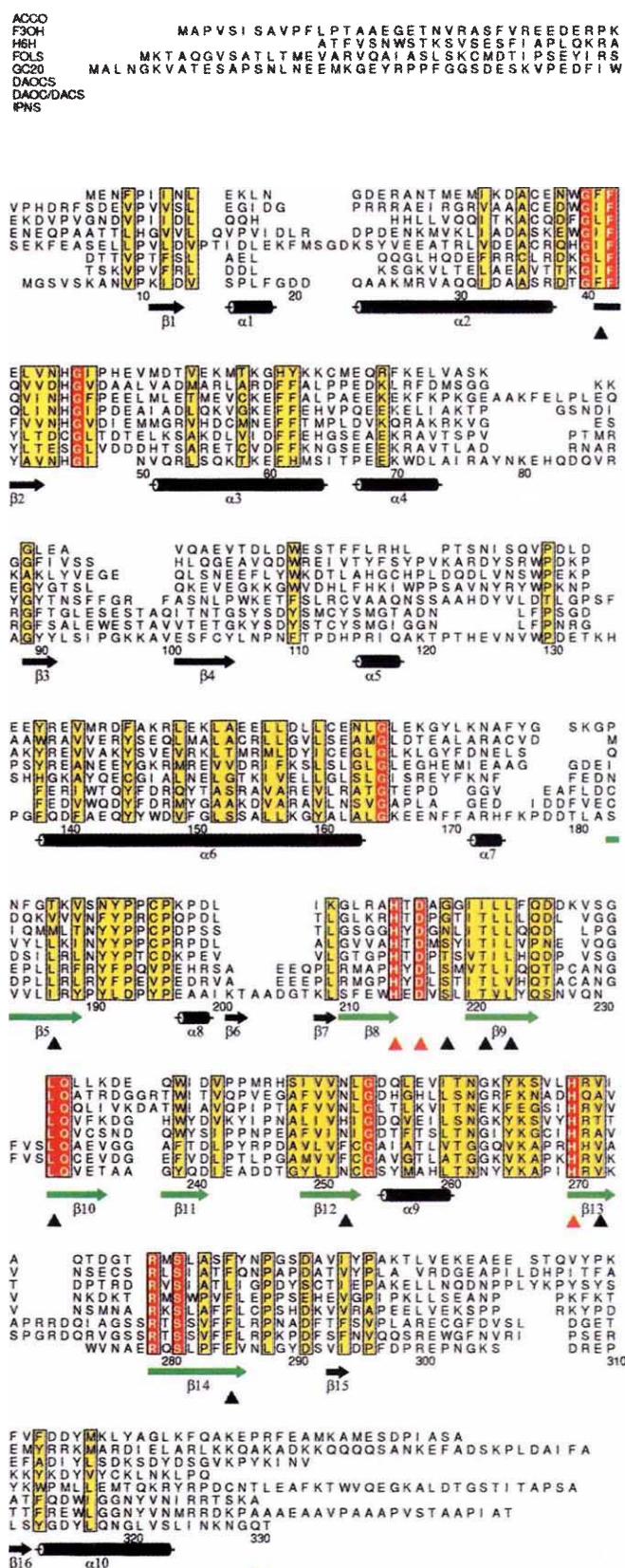
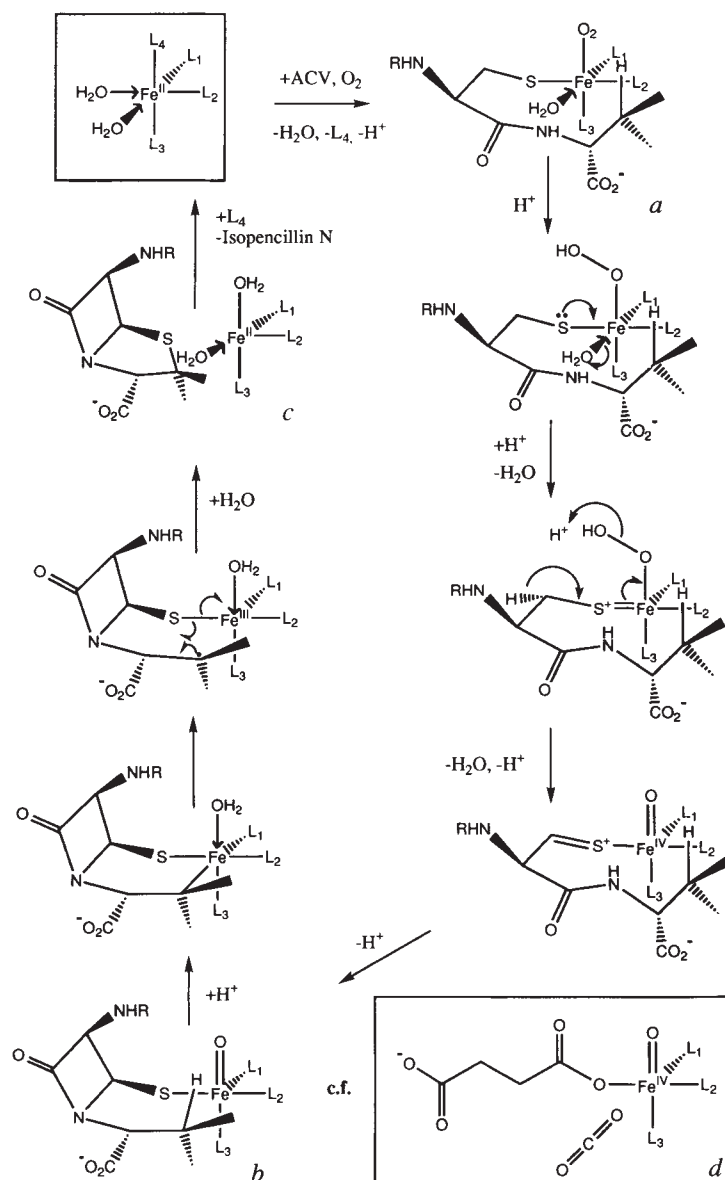


FIG. 2 Sequence comparisons between isopenicillin *N* synthase, 1-aminocyclopropane-1-carboxylic acid oxidase and related 2-oxo-acid-dependent oxygenases. The figure was prepared using ALSCRIPT¹⁷. Numbering follows the sequence of IPNS for which the secondary structures are shown as cylinders (helices) and arrows (β -strands). Identities are shown with red backgrounds, positions at which amino acids with similar physical-chemical properties are conserved are shown in yellow as in Fig. 1*b*. β -Strands 5 and 8–14 which comprise the jelly-roll motif are shown in green. The metal ligands are indicated by red triangles. Other residues with side chains within 8 Å of the metal are indicated by black triangles. ACCO, 1-Aminocyclopropane-1-carboxylate oxidase from tomato (accession number SwissProt P05116); F3OH, flavanone 3- β -hydroxylase from *Zea mays* (Genbank U04434); H6H, hyoscyamine 6- β -hydroxylase from henbane (SwissProt P24397); FOLS, flavanol synthase from *Petunia hybrida* (Genbank S67953); GC20, gibberellin C-20 oxidase from *Cucurbita maxima* (Genbank X73314); DAOCs, deacetoxycephalosporin C synthase from *Streptomyces clavuligerus* (SwissProt P18548); DAOC/DACS, deacetoxycephalosporin C synthase/deacetylcephalosporin C synthase, a bifunctional enzyme from *C. acremonium* (SwissProt P11935); IPNS from *A. nidulans* (SwissProt P05326). For some of the 2-oxo-acid-dependent oxygenases (such as clavaminic acid synthase^{14,15}) preliminary sequence alignments did not indicate sufficient sequence similarity with IPNS to predict the presence of the jelly-roll motif with confidence (see also ref. 14). The conservation of structure throughout the 2-oxo-acid-dependent and related oxygenases will require further structural analyses. Pairwise comparison of the sequences followed by single linkage cluster analysis shows that the proteins all cluster with standard deviation scores >6.0 . Scores over 6.0 indicate that the automatic multiple alignment will be correct within most of the core secondary structures¹⁶. The greatest variations in sequence and hence regions of lowest confidence in the alignment are between residues 1–8, 15–30, 72–137, 168–184, 198–207 and 298–331. It is anticipated that the main differences in tertiary structure between members of the family will be concentrated in these regions.

FIG. 3 Proposed scheme for the overall reaction catalysed by IPNS. L_{1-4} , Protein ligands (His 214, Asp 216, His 270, Gln 330); R, *L*- δ -(α -amino)adipoyl. The scheme shows (a), the two substrates bound to the iron, b, the proposed β -lactam ferryl intermediate and c, the enzyme-product complex with IPN and two water molecules. Mechanistic studies on the conversion of ACV to IPN, using kinetic isotope effects and substrate analogues, have led to the proposal of an enzyme-bound β -lactam intermediate directly attached to the iron centre, at the oxidation state of iv (b). This complex may then react to complete carbon-sulphur bond formation by a free-radical type mechanism^{5,18}. Spectroscopic investigations on the nature of the metal ligands at the active site of IPNS have indicated that the side chains of 2 or 3 histidyl and an aspartyl residue act as metal ligands^{5,27-30}. EPR measurements have been interpreted as showing the presence of a water molecule coordinated to the ferrous iron in an IPNS/ACV/NO complex analogous to a³⁰. Support for the proposed iron-sulphur bond (in a) has come from UV-VIS²⁷ and EXAFS spectroscopy²⁸. Indeed, the active site surrounding the metal-binding site of IPNS is a predominantly hydrophobic cavity, consistent with the generation of a highly reactive intermediate, ligated to the metal by residues that are highly conserved throughout the family, His 214, His 270 and Asp 216 (Fig. 2). The intermediacy of a ferryl species such as b implies a protein-bound ligand (L_4), presumably Gln 330, is released from the iron before its formation. Initial modelling studies indicate substrate binding may induce substantial reorganization at the active site. The presently proposed mechanism for the 2-oxo-acid-dependent oxygenases involves initial formation of an enzyme/iron/2-oxo acid/dioxygen complex, from which a ferryl intermediate d structurally related to b is subsequently formed⁶. The three protein ligands to the metal in this putative complex are probably also the conserved aspartyl and two histidyl residues.



cyclization reactions. Some members have been shown to have a remarkably lax substrate specificity^{15,18}. The determination of the structure of IPNS will help investigations into their catalytic mechanisms and will allow systematic engineering to attempt the enzymatic synthesis of new types of otherwise inaccessible

β -lactams and improve the biosynthetic route to antibiotics of medicinal importance. Studies on the mechanism may also lead to the design of new non-protein catalysts capable of catalysing in a stereoselective manner reactions that are currently synthetically impossible. □

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A cultural exchange

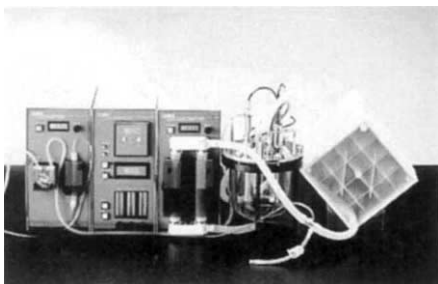
Featuring cell tissue culture, this edition of product review offers a vibrating microtome, enclosed tissue processing, a rotary cell culture system, a microhomogenizer, and a reference guide to cell lines and hybridomas.

LEICA has introduced the new Leica VT 1000 **vibrating knife microtome** (*Reader Service No. 100*). This instrument is designed for sectioning fresh tissue or fixed tissue immersed in a cooled buffer solution to preserve enzymatic activity. It is available in two versions, the semiautomatic 1000 M and the fully automatic 1000 E. The vibrating knife sectioning method produces sections down to a thickness of approximately 0.10 μm , by utilizing motorized knife vibration and knife movement. The manufacturer states that embedding or deep-freezing is no longer necessary before sectioning, thus avoiding tissue damage due to chemical influences, or exposure to high temperature, as well as morphological alterations due to very low temperatures.

The Wimby-03 **microhomogenizer** from Intracel is designed to produce homogenates without destroying the macromolecular structure of biological tissues (*Reader Service No. 101*). This microhomogenizer processes sample sizes as small as 0.2 mg using a fine spring needle, which vibrates between 300 and 2,000 Hz in a microtube. Frequency, amplitude and the duration of processing are adjustable according to the type of tissue and the characteristics required of the homogenate. The unit is designed for quiet operation and occupies only 300 cm² of bench space.

■ Chambers for tissue culture

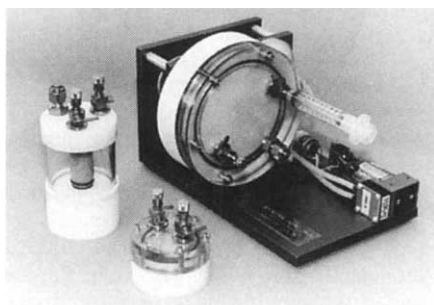
Corning Costar offers the CellCube, a system designed to be fast and compact for high-volume **cell culture production** (*Reader Service No. 102*). CellCube offers polystyrene growth surfaces in a controlled environment, which can be scaled up to meet full production requirements. Each individual plate is fed by a flow control orifice, which distributes media evenly throughout the assembly. Freshly oxygenated media creates a microenvironment for the rapid proliferation of a monolayer of cells on both sides of each plate. The



Corning Costar's CellCube.

company will dedicate one of its scientific staff to the consumer for training, to ensure that CellCube is optimized to the cells and media of a given laboratory.

The **rotary cell culture system** from Cellon enables cultivation of differentiated, three-dimensional cultures which mimic the structure and function of parental tissues (*Reader Service No. 103*).



Cellon's tissue culture system.

The system is designed to promote cell growth to tissue density, matrix formation and cellular differentiation providing a model for studying normal tissue development and transformation processes in neoplastic tumour development.

■ Media

Metachem Diagnostics has announced the availability of MethoCult products, a range of methylcellulose-based **medium for haematopoietic cell culture** (*Reader Service No. 104*). The manufacturer states that there are two key elements to haematopoietic colony assays: the use of a culture medium that maximizes the growth and differentiation of the progenitors of interest and the use of a gelling agent that allows the clonal progeny of a single progenitor cell to stay together. These factors are said to facilitate the recognition and enumeration of distinct colonies. MethoCult is intended to allow better growth of erythroid colonies, as well as allowing granulopoietic colony formation. This methylcellulose-based medium is available for the assay of human and murine primitive haematopoietic cells.

Sigma Cell Culture's new **serum-free, protein-free medium** is a chemically defined medium that supports the fusion, cloning and growth of hybridoma and myeloma cells (*Reader Service No. 105*). Based on Ham's F-12 nutrient mixture with additional amino acids and other components, this medium is designed to require no serum or other supplementation.

It is buffered with MOPS, but does not contain sodium bicarbonate or phenol red. As it lacks protein and phenol red, the medium simplifies the downstream purification of monoclonal antibodies. The serum-free, protein-free medium is sterile, endotoxin tested and hybridoma tested.

■ Cabinets and incubators

Imperial Laboratories has announced the availability of the **modular incubator chamber** (*Reader Service No. 106*). This product allows researchers to culture cells in non-standard gas mixtures, or to utilize mixtures not available from their own incubator. Culture flasks, multiwell plates, Petri dishes and tubes are placed inside the chamber, which is then flushed with the required gas mixture, sealed and placed inside a regular incubator. The manufacturer states that the chamber will hold the gaseous environment for up to 96 h. This should prove useful for researchers interested in studying hypoxia. The chamber also provides a stable environment inside normal CO₂ incubators, buffering cultures from the large fluctuations of temperature and CO₂ levels often experienced with repeated opening of the incubator door. Evaporation from multiwell plates and petri dishes can be greatly reduced by placing them in a humidified modular incubation chamber.

The Shandon Pathcentre has been designed for high-capacity **enclosed tissue processing** (*Reader Service No. 107*). It has a 324-cassette capacity and the ability to extend this with further processing modules. The system is totally enclosed so that no fumes are released into the laboratory during processing. The multilingual programming provides safety and processing efficiency with full on-screen display, on-line help facilities and graphics support to guide and inform the user at every stage.



Pathcentre

MAT has introduced UniMAT, a **compact class II type safety cabinet** for cell culture work (*Reader Service No. 108*). It has been designed to fit into small laboratories and in areas where access is difficult. Design features include an ergonomic layout to reduce operator fatigue when

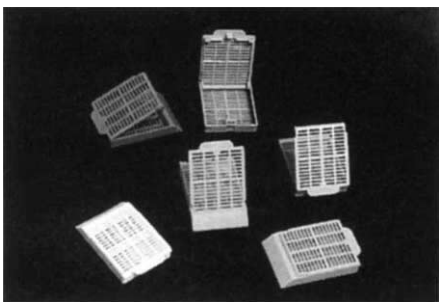


The UniMAT class II type safety cabinet.

carrying out repetitive operations and a sloping front screen to eliminate glare for an improved view of the working area. Laminar downflow over the work surface provides sterile conditions, and the inward airflow through the open front gives a high level of protection to workers. In the event of a breach of the HEPA filters or seals, total negative pressure ensures that unfiltered air cannot escape.

■ Miscellaneous

Globe's disposable tissue cassettes are designed to provide a singular unit for processing, embedding, sectioning and storage of tissue specimens (Reader Service No. 109). The manufacturer has designed these high-density plastic cassettes to allow samples to remain submerged and to allow ample flow for maximum fluid distribution and correct drainage. They are resistant to the effects of microwaving, histological solvents and decalcifiers. These standard



Tissue culture cassettes from Globe.

size cassettes fit in all common base moulds and storage cabinets. The front and sides have a flat surface area for writing any necessary information and 11 different colours are available for easy specimen coding.

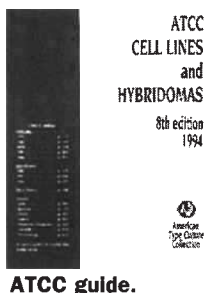
The detection and quantification of reverse transcriptase (RT) in tissue culture supernatants is now possible with the

single-tube RT assay by Amersham Life Science (Reader Service No. 110). Developed specifically for HIV RT, Quant-RT avoids the need for sample preparation and the washing steps typical of traditional RT assays. As with these methods this product relies on the incorporation of a labelled nucleotide into a growing DNA strand to detect RT activity. However, because it is based scintillation proximity assay technology, this technique eliminates the need to separate unincorporated nucleotide. A kit contains a complete set of optimised reagents necessary to perform 200 assays. The method also lends itself to automation in a 96-well microplate format.

New Brunswick has developed AFS BioCommand, a new Windows-based software package for fermentation and cell culture process management (Reader Service No. 111). This software has been designed to manage up to eight fermentors, bioreactors or other instruments — it automates tasks such as logging data, computing real and derived process values, and altering setpoints in response to metabolic changes in culture. Automated setpoint changes can be implemented without having to write a single equation, making this software fast and easy to learn. The information display is designed for side-by-side comparison of several instruments, or several parameters from a single instrument.

■ Catalogues

The American Type Culture Collection cell lines and hybridomas reference guide contains 660 pages with descriptions of approximately 2,400 cell lines and 975 hybridomas (Reader Service No. 112). All entries include recommended growth media, cell line history, references and depositor information. The general index is supplemented by a comprehensive set of special indexes to help locate desired cell lines by species, tissue type, tumour type, monoclonal antibody and depositor/originator. ATCC now provides their culture databases on CD-ROM. The format is cross-platform compatible for DOS, Windows or Macintosh.



ATCC guide.

Clonetics now offers its 1995-96 normal human cell systems catalogue (Reader Service No. 113). The catalogue features various complete, *in vitro*, normal human cell culture systems containing normal human primary cells, optimized serum-free and low-serum growth medium and optimized subculture reagents. All

cells are available as proliferating, in T-flasks or PreSeeded 96-well plates, or cryopreserved (5×10^5 cell per amp). The cell culture systems include renal, endothelial, smooth muscle, skeletal muscle, keratinocyte, fibroblast, melanocyte, mammary and bronchial/tracheal epithelial. Also offered are the NeutralRed bioassay (a complete *in vitro* cytotoxicity kit), growth factors, reagents and system supplements. □

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

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